Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading

# Detection of influenza A virus RNA in birds by optimized Real-Time PCR system

Ilinykh Ph A, Shestopalova EM, Khripko Yu I, Durimanov AG, Sharshov KA, Shestopalov AM

*State Research Center of Virology and Biotechnology Vector, Russia, Novosibirsk region, 630559 Koltsovo*

ARTICLE INFO

*Article history:*
Received 12 March 2010
Received in revised form 7 April 2010
Accepted 10 April 2010
Available online 20 May 2010

*Keywords:*
Influenza A virus RNA
Real-Time PCR
MDCK cell culture
Virus surveillance
Wild birds

ABSTRACT

**Objective:** To evaluate the use of Real-Time PCR system based on specific amplification of matrix protein gene fragment for influenza A virus RNA detection in cloacal swabs from wild birds. **Methods:** Sensitivity, specificity and reproducibility of analysis results were identified. Study of cloacal swabs from wild birds for influenza A virus presence was performed. **Results:** Reproducibility of low concentrations of virus detection in samples by Real-Time PCR was significantly higher than that of detection based on cytopathic effect of viruses grown on MDCK cell culture. **Conclusions:** Real-Time PCR system for influenza A virus RNA detection is developed and applied for virus surveillance study.

## 1. Introduction

Populations of waterbirds are considered to be the main natural reservoir for influenza A viruses. Virus carrying occurs in their organisms preferentially without any symptoms. Highly pathogenic viruses such as H5N1 serotypes can be easily transmitted to poultry during contacts with infected wild bird species. Congestion of poultry creates favourable conditions for rapid distribution of viruses among all livestock population. Highly pathogenic viruses produce pathological statement of birds accompanied by strong features of illness which finally leads to bird's death very often. As a consequence, avian influenza viruses circulating in wild birds correspond the serious danger for poultry keeping as they could kill up to 100% of livestock population and thus causing significant economic loss for poultry farms[1].

Long-distance wild bird migrations promote wide distribution of influenza viruses over different parts and regions of the world[2,3]. The outbreak of influenza infection occurred in 2005 in different regions of Russia may serve as an example of participation of bird migrations at virus distribution. During epizooty in Novosibirsk region in summer, H5N1 virus was isolated from dead domestic birds. Phylogenetic analysis of this virus allowed to presume that it was carried by wild migratory birds from Lake Qinghai (north-western People's Republic of China) to the territory of western Siberia[4].

Virus transmission to such domestic animals as birds and pigs and contact of people with infected animals provide additional possibilities for reassortation of virus genomes and emergence of new variants which can potentially be able to provoke the next pandemia and/or occur to be highly pathogenic for poultry. Therefore it is necessary to obtain the data concerning the properties of virus subtypes persisting in bird population at present for providing the complex of measures against influenza outbreaks among birds and people. Opportune surveillance of viruses circulating in wild birds should be performed for permanent updating of information about virus prevalence in bird populations. Via the high genetic changeability of influenza viruses their constant circulation in nature produces new virus variants. The necessity of systematic renewal of influenza A virus detection systems seems obvious because with the lapse of time they lose the ability of reliable pathogen identification due to reduction of specificity of their components for genetic material of emerging virus strains. Hereupon the main goal of this study was to develop the influenza A virus RNA Real-Time PCR detection system, perform the comparative analysis of influenza A virus detection by Real-Time PCR and virological methods, to estimate the effectiveness of introduced system for influenza A virus surveillance in wild bird populations.

*Corresponding author: Ilinykh Ph A, State Research Center of Virology and Biotechnology Vector, Russia, Novosibirsk region, 630559 Koltsovo.
Tel: +7(383)3367448
Fax: +7(383)3367540
E-mail: ifa83@mail.ru

## 2. Materials and methods

Computer program "CLUSTAL" ("UCD", Ireland) was used for alignment of M protein gene sequences of various influenza A virus subtypes. The selection of primer and probe nucleotide sequences and assessment of their overlapping with M protein gene sequences was performed by "Vector NTI 6.0" program.

Developed Real-Time PCR system characteristics were evaluated using the virus strain A/goose/Krasnoozerskoe/627/2005 (H5N1) (EF205157, EF205164, EF205171, EF205178, EF205185, EF205192, EF205199, EF205206) contained in allantoic fluid as a source material. Its physical titer was measured by negative phosphotungstic acid staining and formed $10^{10}$ virus particles/mL. The sensitivity and reproducibility of Real-Time PCR were compared with influenza virus detection by the instrumentality of the sensitive MDCK cell culture. The similar aliquots (100 μL, 1 000 virus particles/mL) were used for RNA extraction and cell infection. Essential concentration of virions in the samples was obtained by dilution of initial virus-containing allantoic fluid. Mentioned aliquots were carried into the equivalent volume of cell suspension with 200 000 cells/mL. After the samples were cultivated for 7 days, influenza A virus presence was revealed by cytopathic effect visualization. Positive samples were additionally confirmed by hemagglutination test.

To assess the specificity of proposed Real-Time PCR system, A/roody shelduck/Mongolia/632/2007 (H1N1), A/garganey/Altai/1216/2007 (H3N6) (CY049777-CY049784), A/magpie/Mongolia/17/2003 (H4N6), A/duck/Omsk/1822/2006 (H5N1) (CY047472-CY047479), A/garganey/Crimea/26/2008 (H10N6), A/herring gull/Mongolia/454/2008 (H13N8) and B/Malaysia/2506/2004 (CY040449-CY040456) virus strains were used.

Cloacal swabs from *Anatidae*, *Rallidae*, *Ardeidae*, *Podicipedidae* and *Scolopacidae* families were gathered at Malie Chany Lake (Russia, Novosibirsk region) in autumn 2008. The aliquots of collected material were 3 times passaged in 10-day-old, specific-pathogen - free embryonated chicken eggs. After the allantoic fluid was harvested, virus was titrated by hemagglutination test with a 0.5% suspension of chicken red blood cells.

RNA was extracted from cloacal swabs and allantoic fluid using the kit for nucleotide acids isolation (DNA-technology, Russia) according to manufacturer's protocol. cDNA was synthesized from 12 μL of extracted RNA for 1 hour, at 37 ℃ with 100 pmol of random hexaoligonucleotide primer, 200 U of reverse transcriptase M-MuLVRT in 20 μL of buffer (Fermentas, USA). We used 0.12 μmol of each primer (forward: 5′-TCGAAACGTACGTTCTCTCTATC-3′, reverse: 5′-TGTCTTCAGCCATTCCATGAG-3′) and 0.2 pmol of probe (5′-FAM-TCAGGCCCCCTCAAAGCCGA-BHQ1-3′) for Real-Time PCR at "IQ5 Cycler" amplifier (BioRad, USA).

Influenza A virus RNA in allantoic fluid after passaging of field material in chicken embryos was detected by RT-PCR using the primers for nucleoprotein gene[5].

## 3. Results

Full M protein gene sequences of influenza A viruses of various subtypes isolated since 2003 were aligned and analyzed for selection of optimal oligonucleotide sequences of primers and probe for influenza A virus RNA detection. Total number of aligned sequences was 70, all of which were obtained from International Database (GenBank). Figure 1 illustrates the overlapping of one of the sequences with different primers and probes both from references and gene segment of the present study.

The plots reflecting the relation between fluorescence accumulation and amplification cycles were built to characterize the basic parameters of Real-Time PCR detection system - specificity, sensitivity and reproducibility. The possibility of specific identification of influenza A viruses of various subtypes was demonstrated, whereas influenza B virus RNA failed to be detected by this system (Figure 2).

Sensitivity of Real-Time PCR reaction was assessed by measuring of the significantly detected end point and formed at least 100 virus particles per reaction with 100% reproducibility of results (Figure 3). Our system also allowed identifying less concentration of virus - about 10 virus particles per reaction, but reproducibility of these results was only 72.9% (data were not shown in figures). Moreover, the evaluation of virus content after cultivation of samples

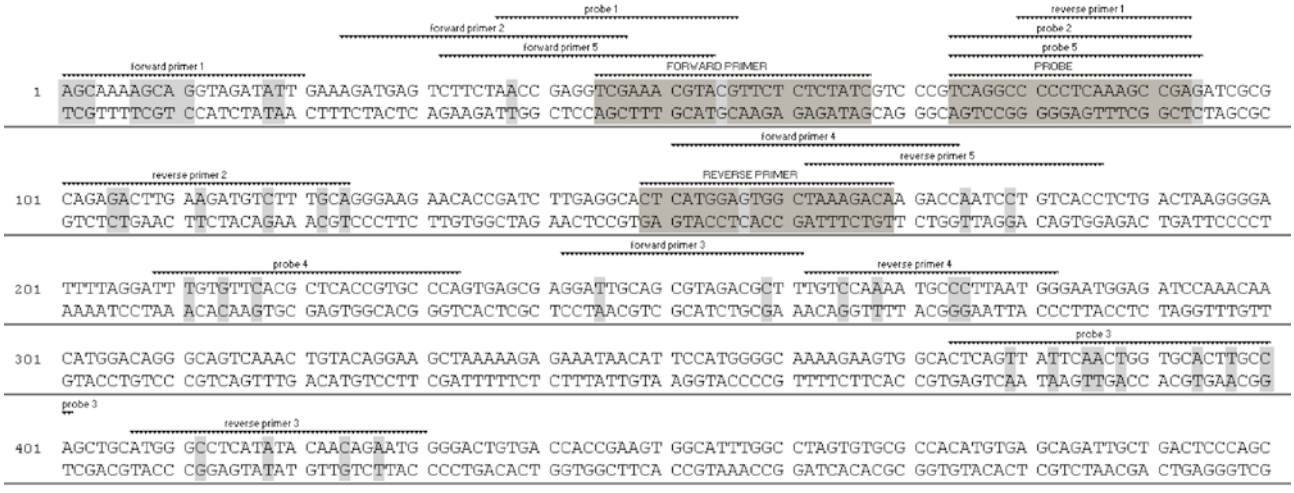


**Figure 1**. Complementary relationships of primers and probes sequences with fragment of M protein gene segment (from -25 to +475 bp) of A/mallard/BC/373/2005 (H5N2) (DQ985167) influenza virus.

The sequences of selected primers and probe are in dark grey (signed with capital letters), possible nucleotide substitutions contained in another M protein gene aligned sequences of various virus strains are in light grey (shown only in places of complementary interactions with primers and probes). The numerals from 1 to 5 indicate primers and probes extracted from references: 1[6], 2[7], 3[8], 4[9], 5[10].

on MDCK cell culture was carried out as well. Absolute initial availability of virus was 100 virus particles in each analyzed sample. Cytopathic effect of virus on cell culture and positive results in hemagglutination test were observed in 10 samples. Total number of samples was 29. So this approach allowed to reveal only 34.5% of total number of wittingly positive samples.

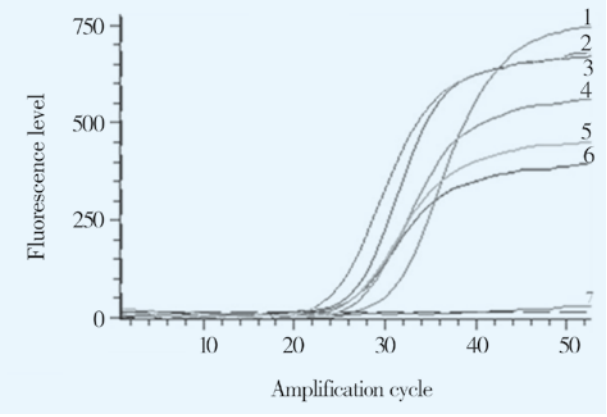


**Figure 2.** Specificity of influenza A virus RNA Real-Time PCR detection system.

The numerals of curves from 1 to 6 indicate different serotypes of influenza A viruses (H10, H1, H4, H13, H5 and H3, respectively), 7 - influenza B virus.

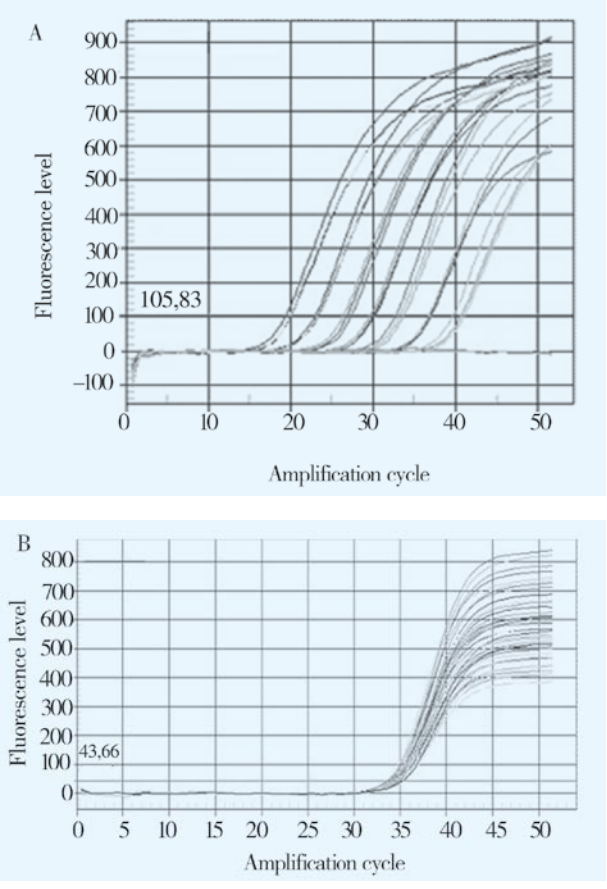


**Figure 3.** Parameters of influenza A virus RNA Real-Time PCR detection system.

A-system sensitivity. The initial concentration of virus was $10^8$ virions/mL, and subsequent dilutions were 10-fold. Each point of titration was repeated for 3 times. Horizontal line on the plot indicates the threshold fluorescence level (105,83).

B- system reproducibility. The concentration of virus was $10^3$ virions/mL, total number of reactions was 48. Horizontal line on the plot indicates the threshold fluorescence level (43,66).

Comparative analysis of influenza A virus attendance in 254 cloacal swabs from waterbirds by means of designed system and other scientific approaches was conducted to estimate a reasonability of offered system usage in virus surveillance study. Influenza A virus RNA in samples was revealed by Real-Time PCR analysis of cloacal swabs and also by simple RT-PCR screening of allantoic fluid after preliminary passaging of field material aliquots in developing chicken embryos which is considered to be the generally accepted method of virus recovery. The results obtained by these two methods are characterized by high coincidence degree (Table 1). Number of positive samples revealed by Real-Time PCR reached 95.8% of virus-containing samples counted after field material cultivation in embryos (23 of 24 positive samples). At the same time, all samples with determined presence of virus genetic material were positive in hemagglutination test. Total percentage of positive samples explored after virus recovery in chicken embryos was 9.4%. This index for Anseriformes birds was 9.9% (24/242).

**Table 1**

Influenza A virus prevalence in cloacal swabs of birds.

| Order | Family | Total number of samples | Number of positive samples | |
|---|---|---|---|---|
| *Anseriformes* | *Anatidae* | 242 | 23 | 24 |
| *Gruiformes* | *Rallidae* | 9 | 0 | 0 |
| *Ciconiformes* | *Ardeidae* | 1 | 0 | 0 |
| *Podicipediformes* | *Podicipedidae* | 1 | 0 | 0 |
| *Charadriiformes* | *Scolopacidae* | 1 | 0 | 0 |
| | Total | 254 | 23* | 24** |

*Real-Time PCR detection of influenza A virus RNA in cloacal swabs; **RT-PCR detection of influenza A virus RNA in allantoic fluid after passaging of cloacal swabs aliquots in chicken embryos.

## 4. Disc;ussion

To create the optimal primers and probe with nucleotide sequences corresponding to the newly emerged avian influenza virus strains, we aligned tens of M protein gene segment sequences of various viruses, compared them and found conservative regions suitable for short oligonucleotide's design for the system of Real-Time PCR detection of influenza A viral RNA. Structures of molecular components of test-systems for biomaterial screening for influenza A virus RNA from several references[6-10] were also compared with aligned sequences that allowed to reveal all nucleotide mismatches between them. The results of sequence analysis show that some primers and probes previously used for influenza A virus diagnostics should be updated due to the large number of nucleotide substitutions in appropriate genome regions of influenza A virus strains isolated in last several years.

As a stepwise incremental process, the measurement of basic parameters characterizing the adequacy of offered system for routine influenza A virus surveillance in biological samples was then performed. Results of viral genetic material detection in samples of allantoic fluid containing H1N1, H3N6, H4N6, H5N1, H10N6, H13N8 or influenza B virus suggested our Real-Time PCR test-system is fully

correspond to specificity criteria as it allowed to reveal various serotypes of influenza A viruses and was absolutely ineligible for influenza B virus RNA identification in biological samples. Another parameter of PCR reaction efficacy being assessed was sensitivity of virus detection. Our experiments demonstrated that sensitivity threshold of Real-Time PCR test-system corresponds to 100 viral genome RNA copies per detecting reaction at least. Sensitivity limit for Real-Time PCR method for its employment in influenza A virus surveillance study varies from 10 to 100 and more RNA copies per reaction[6,8,9]. Thus sensitivity level of introduced Real-Time PCR detection system fit the results of application of this method in diagnostic research.

System reproducibility was analyzed by screening the samples with low virion concentration equaled to 1 000 virus particles/mL. Moreover, the results of two methods of virus detection were compared. Real-Time PCR method showed absolute reproducibility of revealing of positive samples at these low meanings of virus content being used in experiment. At the same time, visual fixation of influenza A virus cytopathic effect after its grown on MDCK cell culture with subsequent confirmation of virus-contaminated specimens by hemagglutination inhibition test let us identify only one-third of the whole set of virus-positive dilutions of infected allantoic fluid. These data provide strong evidence that reproducibility of detection of virus in low concentration by Real-Time PCR is far higher than that of the virus presence determination using MDCK cell culture.

Comparison of our Real-Time PCR system with conventionally used method of virus propagating in 10-day-old eggs before its nucleotide material detection in routine influenza A virus monitoring in natural wild bird's populations pointed at significant similarity of results generated by these two approaches. Thereby the use of introduced system for influenza A virus surveillance obviously leads to substantial time economy in comparison with primary passaging of collected field samples in chicken embryos with subsequent PCR-analysis. Moreover, this method produces the quite insignificant part of false-negative results among total number of analyzable samples.

The endogenous level of influenza A virus infection in different wild bird populations varies from 0 to 10.9%. Specimens of *Anseriformes* order are universally recognized champions since virus carrying may reach up to 15.2% in their populations[1,3,11-14]. During collection of cloacal swabs Anseriformes birds were just prevalent. The sampling of birds belonging to other orders was minor. Total percentage of positive samples explored after virus recovery in chicken embryos was 9.4%. This index for Anseriformes birds was 9.9%.

Hereby influenza A virus RNA Real-Time PCR detection test-system with necessary values of specificity, sensitivity and reproducibility was designed. Comparative analysis of reproducibility of this system application and cultivation of sample aliquots in MDCK cells with subsequent influenza A virus detection by serological methods was conducted. Extracted data denote advantage of available influenza A virus genome-targeted system for detection of small quantity of virus material corresponding to sensitivity level for Real-Time PCR. The results of practical approbation of our system give us a possibility to recommend it for diagnostic research of influenza A virus environmental circulation.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

The authors thank Dr. Ternovoy V. A. (State Research Center of Virology and Biotechnology Vector) for critical review of the manuscript, Dr. Ryabchikova E. I. (Institute of Chemical Biology and Fundamental Medicine) for help in determination of physical titer of A/goose/Krasnoozerskoe/627/2005 (H5N1) virus strain, and Dr. Nechaeva E.A. (State Research Center of Virology and Biotechnology Vector) for kindly providing of B/Malaysia/2506/2004 virus strain.

## References

[1]Alexander DJ. An overview of the epidemiology of avian influenza. *Vaccine* 2007; **25**: 5637-44.
[2]Gilbert M, Xiao X, Domenech J, Lubroth J, Martin V, Slingenbergh J. Anatidae migration in the Western Palearctic and spread of highly pathogenic avian influenza H5N1 virus. *Emerg Infect Dis* 2006; **12**: 1650-6.
[3]Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus ADME, Fouchier RAM. Global patterns of influenza A virus in wild birds. *Science* 2006; **312**: 384-8.
[4]Shestopalov AM, Durimanov AG, Evseenko VA, Ternovoi VA, Rassadkin YN, Razumova YV, et al. H5N1 influenza virus, domestic birds, Western Siberia, Russia. *Emerg Infect Dis* 2006; **12**: 1167-9.
[5]Lee MS, Chang PC, Shien JH, Cheng MC, Shieh HK. Identification and subtyping of avian influenza viruses by reverse transcription-PCR. *J Virol Methods* 2001; **97**: 13-22.
[6]Krafft AE, Russell KL, Hawksworth AW, McCall S, Irvine M, Daum LT, et al. Evaluation of PCR testing of ethanol-fixed nasal swab specimens as an augmented surveillance strategy for influenza virus and adenovirus identification. *J Clin Microbiol* 2005; **43**: 1768-75.
[7]Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 2002; **40**: 3256-60.
[8]van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. *J Clin Microbiol* 2001; **39**: 196-200.
[9]Watzinger F, Suda M, Preuner S, Baumgartinger R, Ebner K, Baskova L, et al. Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. *J Clin Microbiol* 2004; **42**: 5189-98.
[10]Whiley DM, Sloots TP. A 5′-nuclease real-time reverse transcriptase-polymerase chain reaction assay for the detection of a broad range of influenza A subtypes, including H5N1. *Diagn Microbiol Infect Dis* 2005; **53**: 335-7.
[11]Ip HS, Flint PL, Franson JC, Dusek RJ, Derksen DV, Gill Jr RE, et al. Prevalence of influenza A viruses in wild migratory birds in Alaska: patterns of variation in detection at a crossroads of intercontinental flyways. *Virol J* 2008; **5**: 71.
[12]Lindh E, Huovilainen A, Rätti O, Ek-Kommonen C, Sironen T, Huhtamo E, et al. Orthomyxo-, paramyxo- and flavivirus infections in wild waterfowl in Finland. *Virol J* 2008; **5**: 35.
[13]Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, et al. Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS Pathog* 2007; **3**: 0630-8.
[14]Winker K, Spackman E, Swayne DE. Rarity of influenza A virus in spring shorebirds, Southern Alaska. *Emerg Infect Dis* 2008; **14**: 1314-6.