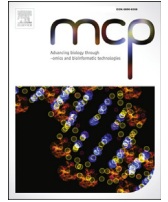




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Development of a realtime RT-PCR assay for the rapid detection of influenza A(H2) viruses



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ABSTRACT

Influenza and other acute respiratory infections are of great concern for public health, causing excessive morbidity and mortality throughout the world. Influenza virus A(H2N2), which caused a pandemic of so called “Asian flu” in 1957 was expelled from the human population by the new pandemic virus subtype H3N2 in 1968, however, influenza A(H2) viruses continue to circulate in wild birds and poultry. The lack of immunity in human population and the continued circulation of influenza A(H2) among animals makes emergence of a new pandemic virus possible. One of the basic techniques of molecular diagnostics of infectious diseases is the realtime polymerase chain reaction (PCR). The aim of this work was to design oligonucleotide primers and probes for the rapid detection of influenza A virus subtype H2 by realtime reverse transcription - polymerase chain reaction (rRT-PCR).

Analysis of 539 sequences of influenza A(H2N2) virus hemagglutinin gene from GISAID EpiFlu database revealed conservative regions suitable for use as binding sites for primers and probes. 191 probes were designed and 2 sets of primers and probes (H2-1 and H2-2) were selected for further experimental evaluation. Detection limit of RT-PCR system was 50 copies of DNA per 25 μ l reaction when 10-fold dilutions of pCI-neo-H2 plasmid used as template. Analytical specificity of selected sets of primers and probes were tested on wide range of influenza strains and non-influenza respiratory viruses. H2-2 set found to have insufficient specificity detecting seasonal influenza A(H1N1) viruses and was excluded from further analysis. Analytical sensitivity was further tested on vaccine strain A/17/California/66/395 (H2N2) and A/Japan/305/1957 (H2N2), limit of detection for primers-probe set H2-1 was 3.2 (CI95%: 3.07–3.48) lg EID₅₀/ml.

Designed primers and probes for the realtime RT-PCR universal detection of influenza A(H2) viruses could be used in clinical trials of vaccines against influenza A(H2) and screening for H2 in cases of unsubtypeable influenza A in humans.

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1. Introduction

Influenza virus is an enveloped virus with segmented (–)ssRNA genome belonging to family Orthomyxoviridae. Family comprises 5 genera recognized by ICTV: Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus and Isavirus, and 2 proposed genera

– Quaranzavirus and Influenzavirus D. Influenza A viruses are most variable of all orthomyxoviruses being the cause of influenza epidemics and pandemics. Now 18 hemagglutinin (HA) and 11 neuraminidase (NA) subtypes of influenza A virus are recognized. Most of them have waterfowl as a host. Only influenza viruses of H1, H2 and H3 subtypes are known to cause pandemics and circulate in human population for considerable time. Only sporadic cases of infection on human-animal interface by influenza A(H5), A(H6), A(H7), A(H9) and A(H10) were observed.

Over the past 100 years mankind survived five pandemics of influenza A: from devastating “Spanish flu” in 1918 taking more

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than 50 million lives to “Swine flu” in 2009 with much less mortality and economic losses. In so called “Asian flu” pandemics in 1957–1958, caused by influenza A(H2N2) virus around 1–2 million lethal cases were registered [1]. This virus emerged in humans following antigenic shift resulting from reassortment between circulating human H1N1 influenza virus and avian H2N2 influenza viruses. This reassortant 1957 pandemic viruses possessed the HA (4), NA (6), and PB1(2) gene segments of an avian H2N2 virus while the remaining five were of human H1N1 virus origin [2]. Pandemic started in Northern China in February 1957 [3] and in several weeks spread to South East Asia. In USSR first cases were registered in Central Asia (Tashkent, Dushanbe, Almaty), first wave of pandemic reached European part of USSR in the end of May 1957 [4]. Mortality rate in Leningrad in 1957–1959 was near 1.5% of population [5]. In 1968 influenza A(H2N2) virus was superseded by influenza A(H3N2) virus.

Expelled from human population in 1968 influenza A(H2N2) viruses continue to circulate in wild birds and poultry [6–8]. Two phylogenetic lineages of influenza A(H2) viruses exist: Eurasian and American. Avian influenza A(H2) viruses of Eurasian lineage are phylogenetically and antigenically close to 1957 pandemic strain [6,9]. Influenza A(H2) strains of American lineage were isolated from swine. It was shown that swine H2 viruses have altered receptor specificity recognizing α 2-6 sialic acids and are capable of contact transmission from swine to swine [9].

Susceptibility to infection is determined by various host factors including presence or absence of specific antibodies produced as a result of previous infection or vaccination. Influenza A(H2N2) virus has not circulated in humans for over 50 years, vaccination stopped in the late 1960s, so now the population is almost immunologically naïve to influenza A(H2) infection. People born after 1968 are seronegative to influenza A(H2) [1,10,11]. The lack of immunity and the continued circulation of influenza A (H2) in wild birds, poultry and swine raises a serious concern for the emergence of a new pandemic.

To date conventional RT-PCR technique for detection of A(H2N2) viruses has been established [12]. Conventional RT-PCR is more laborious and error-prone due to risk of contamination by PCR products. Also there are realtime RT-PCR assays for wide spectrum of influenza A viruses, including H2 strains [13].

The aim of the current study was to develop and evaluate realtime RT-PCR-based assay for rapid detection of influenza A(H2) viruses.

2. Materials and methods

2.1. Virus strains

Influenza viruses A/Japan/305/1957 (H2N2), A/Saint-Petersburg/RII66/2009 (H1N1), A/seal/Caspian sea/01/2000 (H7N7) and A/herring gull/Atyrau/2186/2007 (H11N2) and LAIV A/17/California/66/395 (H2N2) were stored, propagated using 10-

day-old chicken embryos according to the method described previously [14]. Influenza viruses A/Nizhniy Novgorod/RII01/2013 (H1N1pdm09) and A/Saint-Petersburg/RII06/2013 (H3N2) were propagated on MDCK cells. 50% embryonic infective dose (EID50) was calculated using Reed-Muench method [15].

2.2. Sequence analysis

539 full-length (>1700 nt, duplicates removed) sequences of H2 hemagglutinin gene (433–avian, 91–human, 3–other mammals, 12–environmental) were obtained from EpiFlu GISAID database (See Table A.1). Shannon entropy as an estimate of sequence conservation was calculated using Python script. Python script is available on GitHub (<https://github.com/MVGE-lab/shannon-entropy-calculator>). Local minima in distribution of Shannon entropy were selected for TaqMan primers and probes design.

2.3. Primer and probe design

Primers and probes were designed using Beacon Designer v7.70 (Premier Biosoft). The following requirements were used for TaqMan probes design: probe length 18–25 nt, ΔG_{\max} (hairpin) –6.0 kcal/mol, ΔG_{\max} (dimer) –10.0 kcal/mol, maximum repeat length 5 nt. Probe rating was calculated using the formula:

$$\text{Probe rating} = 100 \times \left[1 - \sqrt{\frac{1}{N} \sum_{n=1}^N \left(\frac{P_n - T_n}{V_n} \right)^2} \right]$$

N – number of parameters

P_n – calculated value of parameter n

T_n – set value of parameter n

V_n – permissible limit of variation of parameter n

Only probes with rating from 80 to 100 were selected for further analysis. For all selected probes Shannon entropy values were calculated using Python script. Sequence conservation of probes was visualized using WebLogo [16,17]. The specificity of primers and probes was verified by Primer BLAST [18] and BLAST [19], respectively. The probes were labeled with FAM and JOE at the 5'-end and BHQ1 and BHQ2 at the 3'-end. The primers and probe used are listed in Table 1.

2.4. RNA extraction and rRT-PCR

Total RNA was extracted from allantoic fluid and cell culture supernatant using QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. rRT-PCR was performed using AgPathID One-Step RT-PCR Kit (Ambion, USA). 25- μ l reaction mixture contained 12.5 μ l 2x reaction premix, 0.5 μ l forward primer

Table 1
Primers and probes for identification of influenza A(H2) viruses.

| Primer/probe | Sequence (5'-3') | Length | Tm ^a , °C | Binding site |
|--------------------------------|--|--------|----------------------|--------------|
| H2-P1 | FAM -TGGACACAGCATAACAACACTGG- BHQ1 | 23 | 67,37 | 409–431 |
| H2-F1 | AAGATTCTGCCCAAAGAT | 18 | 57,80 | 388–405 |
| H2-R1 | TCCGCTCGTATTGTGTGA | 18 | 60,05 | 532–549 |
| Amplicon length: 162 bp | | | | |
| H2-P2 | JOE -CATGGAAGTCAAGTGTCTCTCA- BHQ2 | 23 | 66,09 | 1354–1332 |
| H2-F2 | CTTCTAGTCCTGATGGAA | 18 | 56,5 | 1300–1317 |
| H2-R2 | GCATTCTGACTTTTCATAC | 20 | 56,72 | 1368–1387 |
| Amplicon length: 82 bp | | | | |

^a Tm is calculated using Beacon Designer (oligo concentration: 200 nM, monovalent ion concentration: 50 mM, free magnesium concentration: 3 mM).

(20 pmol/μl), 0.5 μl reverse primer (20 pmol/μl), 0.5 μl probe (5 pmol/μl), 1 μl template and 10 μl of DEPC-treated water. Reactions were performed on Bio-Rad CFX96 realtime PCR instrument (Bio-Rad, USA) using the following cycling conditions: 30 min at 50 °C, 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 30 s at 55 °C. DEPC-treated water was used as a no template control (NTC). The data was analyzed using Bio-Rad CFX Manager 2.1 software. Ct cut-off value was set 38.

2.5. Capillary sequencing

PCR amplicons were analyzed by agarose electrophoresis, agarose gels were documented using ChemiDoc MP gel documentation system (Bio-Rad). DNA was extracted from agarose gels by QIAquick Gel Extraction Kit (QIAGEN) according to manufacturer's instructions. Concentration of DNA was assessed using NanoDrop spectrophotometer. Capillary sequencing was performed on ABI 3130 genetic analyzer using BigDye Terminator v3.1 kit (Thermo).

2.6. PCR efficiency evaluation

10-fold serial dilutions of pCI-neo expression vector containing H2 HA Gene (FR-576) plasmid DNA were used as the templates for RT-PCR to determine the standard curve and evaluate PCR efficiency of novel assay. rRT-PCR efficiency was evaluated using 10-fold serial dilutions of influenza A/17/California/66/395 (H2N2) virus preparation with known infectious titer.

2.7. Sensitivity test

Limit of detection (LOD) was defined as the highest dilution at which 95% of positive samples were detected. 5-fold serial dilutions of allantoic fluid containing A/Japan/305/1957 (H2N2) with infectious titer 1.39×10^9 EID₅₀/ml in 20 replicates were used for LOD estimation. Then five 2-fold dilutions covering the range from 2.22 to 3.42 lg EID₅₀/ml were tested and probit analysis was conducted using Statgraphics Centurion XVI (StatPoint Technologies, Inc.).

Three 5-fold dilutions of A/Japan/305/1957 (H2N2) virus in phosphate-buffered saline (PBS) and influenza and non-influenza respiratory viruses PCR-negative nasopharyngeal swab collected in virus transport medium (Copan VTM) spiked with the same virus dose were prepared to test the performance of RT-PCR assay on artificial human specimens.

2.8. Specificity test

The specificity of novel assay was evaluated using influenza viruses of various subtypes from Research Institute of Influenza virus collection (A/Saint-Petersburg/RII66/2009 (H1N1), A/Saint-Petersburg/38/2008 (H1N1), A/Saint-Petersburg/95/2008 (H1N1), A/Moscow/52/2001 (H1N1), A/Nizhniy Novgorod/RII01/2013 (H1N1pdm09), A/Saint-Petersburg/RII06/2013 (H3N2), A/seal/Caspian sea/01/2000 (H7N7), A/herring gull/Atyrau/2186/2007 (H11N2), C/Taylor/47)) and CDC influenza Real-time RT-PCR positive controls - CDC Influenza A/H5N1 (Asian Lineage) Real-Time RT-PCR Positive Control with Human Cell Material (RUO) (Catalog No. VA2715) containing BPL-inactivated A/Vietnam/1203/2004 (H5N1), CDC Influenza A/H7 (Eurasian Lineage) Positive Control (EuH7PC) (RUO) (Catalog No. KK0818), containing BPL-inactivated A/Anhui/1/2013 (H7N9) and CDC Pooled Influenza Positive Control (RUO) (-PIPC) (Catalog No. VA2716), containing mix of influenza B virus and, influenza A/H1N1pdm09, A/H1N1 and A/H3N2 viruses.

Also specificity of novel assay was tested on common non-influenza respiratory viruses (hRSV subtype A (strain A2), hRSV subtype A (strain 9320), human parainfluenza virus type 2 (HPIV2/

Saint-Petersburg/10568/2011) and human parainfluenza virus type 3 (HPIV3/Saint-Petersburg/123/2014), adenovirus serotype 1 (type C) from Research Institute of Influenza virus collection. Finally specificity of developed RT-PCR assays was tested on clinical samples positive for influenza A(H3N2), influenza A(H1N1)pdm09, influenza B and various non-influenza respiratory viruses (hRSV, adenovirus, hPIV, rhinovirus, bocavirus, coronavirus and human metapneumovirus) from primary respiratory specimen biobank of RII.

3. Results

3.1. Primer and probe design

539 full-length sequences of H2 hemagglutinin gene (433–avian, 91–human, 3–other mammals, 12–environmental) were obtained from EpiFlu GISAID database (See Suppl. 1) and aligned using MAFFT algorithm (FFT-NS-2) [20]. Shannon entropy (H) was considered as an estimate of sequence conservation. H value can range from 0 (for absolutely conserved positions) to 1.386 (for positions with equal (0.25) substitution rates for all four nucleotides). Both Shannon entropy of each position ($H_{n=1}$) and mean Shannon entropy of a sliding frame of 22 positions ($H_{n=22}$) across HA sequence were calculated. Frame size was adjusted to average primer and/or probe length (22 nt). Local minima in distribution of Shannon entropy $H_{n=22}$ were selected for TaqMan probes design. 191 TaqMan probes covering selected regions were designed. Probes were checked for specificity in silico using BLAST 2.2.28 + against non-H2 influenza A viruses and human genomes (Human genomic plus transcript, G + T database). Probes aligning with non-H2 influenza A virus sequences were excluded from analysis. Probes aligning with human genome sequences were excluded from analysis if query cover in BLAST 2.2.28 + exceeded 70%. Occasional sequence homology with human genome (query cover 70% and less) indicated that special attention should be paid to design of primers to be used with this probe in one assay.

Shannon entropy distribution along the probes sequences was evaluated. As TaqMan technology is based on 5'-end reporter cleavage by polymerase, probes with little sequence conservation in 5'-end were excluded from further analysis. Finally two probes candidates were selected: H2-1 and H2-2, for which WebLogos were made (Fig. 1). FAM and JOE dyes and BHQ-1 and BHQ-2 quenchers were chosen for selected probes.

The following criteria were used for primer design: primer melting temperature should be 5–10 °C lower than probe melting temperature, difference in melting temperature between forward and reverse primer should not exceed 3 °C, amplicon length should be 70–200 bp, distance between primer 3'-end and probe 5'-end should not be longer than 20 nt. Primers H2-F1/H2-R1 and H2-F2/H2-R2 were designed (See Table 1). Primers specificity was tested in silico using Primer BLAST [18], against all sequences excluding influenza A/H2.

3.2. Standard curve for H2 realtime RT-PCR assay

10-fold serial dilutions of pCI-neo plasmid DNA encoding HA gene of influenza A/Japan/305/1957 (H2N2) virus were used to evaluate PCR efficiency of H2-1 and H2-2 primer and probe sets. For set H2-1 the equation used to generate the standard curve was $y = -3.320x + 11.57$, the linear regression coefficient (R^2) was 0.9935, and the amplification efficiency was 100%. For set H2-2 the equation used to generate the standard curve was $y = -3.171x + 16.87$, the linear regression coefficient (R^2) was 0.9842, and the amplification efficiency (E) was 106% (See Fig. 2A).

Detection limit was calculated to be 1.2×10^{-8} ng/μl (50 copies

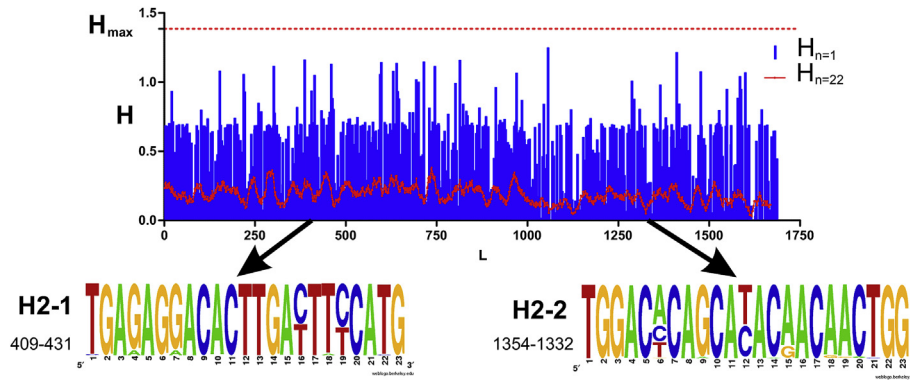


Fig. 1. Shannon entropy (H) distribution across H2 hemagglutinin sequence and web-logos for two selected probe binding sites in local H-value minima.

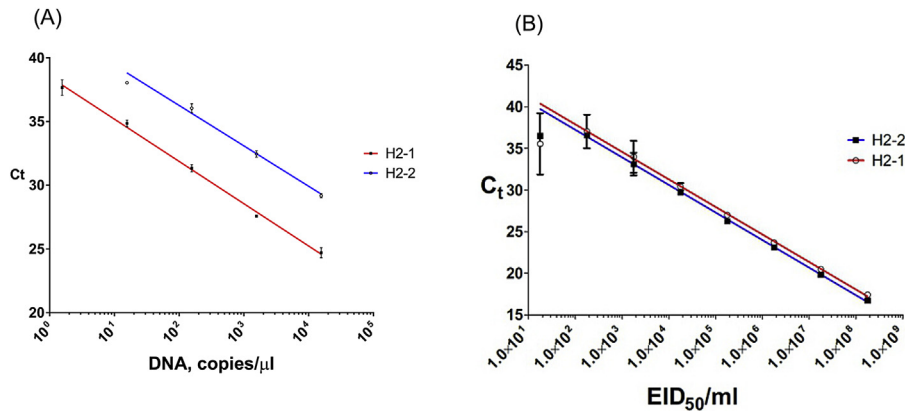


Fig. 2. Standard curves to evaluate efficiency of RT-PCR on pCI-neo-H2 plasmid (A) and A/17/California/66/395 virus (B) using H2-1 and H2-2 primers/probes sets.

of DNA template in 25 μ l reaction volume). To evaluate RT-PCR efficiency 10-fold serial dilutions of influenza A/17/California/66/395 (H2N2) virus (infectious titer 1.78×10^9 EID₅₀/ml) were used. For set H2-1 the equation used to generate the standard curve was $y = -3.306x + 44.50$, the linear regression coefficient (R^2) was 0.9995, and the amplification efficiency was 100.7%. For set H2-2 the equation used to generate the standard curve was $y = -3.311x + 43.86$, the linear regression coefficient (R^2) was 0.9996, and the amplification efficiency (E) was 100.5% (See Fig. 2B).

3.3. Analytical sensitivity

5-fold serial dilutions of A/Japan/305/1957 (H2N2) virus were used to estimate novel assay analytical sensitivity. Probit analysis was used to determine LOD. Limit of detection for set H2-1 (LOD₁) was 3.2 (CI95%: 3.07–3.48) lg EID₅₀/ml, (See Fig. 3B).

In order to test possible interference of human material and virus transport medium with assay performance we compared RT-PCR results obtained on three 5-fold dilutions of A/Japan/305/1957 (H2N2) virus in PBS and clinical specimen spiked with the same

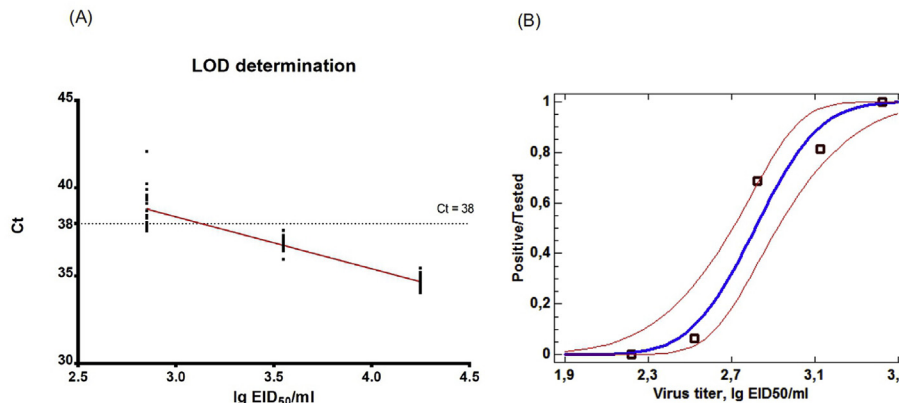


Fig. 3. (A) Determination of limit of detection of rRT-PCR H2-1 assay. (B) Number of positives versus the virus titre of influenza A/Japan/305/1957 (H2N2) virus for RT-PCR H2-1 assay and a regression curve determined by probit regression analysis.

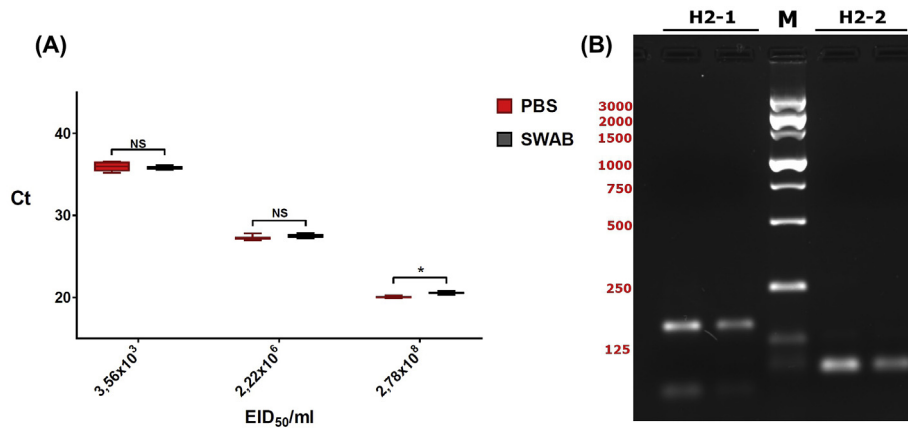


Fig. 4. (A) Comparison of RT-PCR H2-1 assay performance on influenza A/Japan/305/1957 (H2N2) virus dilutions (PBS) and artificial human specimens spiked with A/Japan/305/1957 (H2N2) virus (SWAB). NS – no significance, * – $P < 0.0001$. (B) Agarose electrophoresis of rRT-PCR amplification products of LAIV A/17/California/66/395 (H2N2) using H2-1 and H2-2 primer sets.

amount of virus. Significant retardation of Ct values ($p < 0.0001$) was observed only in samples containing 2.78×10^8 EID₅₀/ml of influenza virus. The results are shown in Fig. 4A.

3.4. Amplicon confirmation by capillary sequencing

Predicted PCR amplicon length for H2-1 and H2-2 primer sets was confirmed by agarose gel electrophoresis (See Fig. 4B). Partial capillary sequencing and sequence alignment further confirmed amplicon identity.

3.5. Analytical specificity

The specificity of novel rRT-PCR assay was tested using influenza viruses of various subtypes from RII virus collection and CDC real-time PCR positive controls and non-influenza respiratory viruses (see Table 2). Both H2-1 and H2-2 primers/probe sets showed high specificity for influenza A(H2) viruses, yielding no fluorescence

signal from samples containing common human influenza A viruses and emerging influenza viruses like influenza A(H5N1) and A(H7N9). H2-2 set demonstrated weak near cut-off fluorescent signal on some human seasonal influenza A(H1N1) influenza virus and pooled influenza positive control (PIPC). Finally, specificity of developed RT-PCR assays was tested on clinical samples positive for influenza A(H3N2), influenza A(H1N1)pdm09, influenza B and various non-influenza respiratory viruses (hRSV, adenovirus, hPIV, rhinovirus, bocavirus, coronavirus and human metapneumovirus) from primary respiratory specimen biobank of RII demonstrating no false positive results. Based on analytical specificity H2-2 was considered to perform non-satisfactory.

4. Discussion

Influenza A(H2N2) virus can infect various host species including waterfowl, swine and humans. In 1968 influenza A(H2N2) viruses were superseded from circulation in humans by

Table 2
Analytical specificity of rRT-PCR assay.

| Virus/Sample | Subtype | H2 rRT-PCR assay | | CDC rRT-PCR assay | | | | | |
|--|-----------------------------|------------------|------|-------------------|----|---------|----|----|----|
| | | H2-1 | H2-2 | InfA | H1 | H1pdm09 | H3 | H5 | H7 |
| Influenza viruses | | | | | | | | | |
| A/Japan/305/1957 | H2N2 | + | + | + | NT | NT | NT | NT | NT |
| A/17/California/66/395 | H2N2 | + | + | + | NT | NT | NT | NT | NT |
| Pooled influenza positive control (PIPC) | InfB, H1N1pdm09, H1N1, H3N2 | – | + | + | + | + | – | – | – |
| A/Nizhniy Novgorod/RII01/2013 | H1N1pdm09 | – | – | + | – | + | – | – | – |
| A/Saint-Petersburg/RII06/2013 | H3N2 | – | – | + | – | – | + | – | – |
| A/Saint-Petersburg/RII66/2009 | H1N1 | – | ± | + | – | – | – | – | – |
| A/Vietnam/1203/2004 ^a | H5N1 | – | – | + | – | – | – | + | – |
| A/Anhui/1/2013 ^a | H7N9 | – | – | + | – | – | – | – | + |
| A/seal/Caspian sea/01/2000 | H7N7 | – | – | + | – | – | – | – | + |
| A/herring gull/Atyrau/2186/2007 | H11N2 | – | – | + | – | – | – | – | – |
| A/Saint-Petersburg/38/2008 | H1N1 | – | NT | + | + | – | – | – | – |
| A/Saint-Petersburg/95/2008 | H1N1 | – | NT | + | + | – | – | – | – |
| A/Saint-Petersburg/52/2001 | H1N1 | – | NT | + | + | – | – | – | – |
| C/Taylor/47 | InfC | – | NT | – | – | – | – | – | – |
| Non-influenza respiratory viruses | | | | | | | | | |
| hRSVA strain A2 | hRSVA | – | NT | – | – | – | – | – | – |
| hRSVB strain 9320 | hRSVB | – | NT | – | – | – | – | – | – |
| HPIV2/Saint-Petersburg/10568/2011 | hPIV2 | – | NT | – | – | – | – | – | – |
| HPIV3/Saint-Petersburg/123/2014 | hPIV3 | – | NT | – | – | – | – | – | – |
| Human adenovirus serotype 1 (type C) | | – | NT | – | – | – | – | – | – |

NT – not tested.

^a Component of CDC real-time RT-PCR positive controls.

influenza A(H3N2) viruses. Since that time no human cases of influenza A(H2N2) infection were reported. Yet evidences of A(H2N2) virus infection in wild birds and poultry are regularly registered. Most of humans born after 1968 are immunologically naïve to influenza A(H2N2) virus. It raises a concern that influenza A(H2N2) can be a potentially pandemic virus. Influenza pandemic preparedness measures include development of rapid diagnostic tests for potentially pandemic viruses. Real-time PCR is a state-of-the-art technology in influenza diagnostics. To date in-house conventional RT-PCR for detection of A(H2N2) viruses has been developed [12]. Conventional PCR has many disadvantages in diagnostic use due to laboriousness, high risk of contamination and long turnaround time. Several protocols for influenza subtyping using conventional PCR were published [21,22]. Recently, Hoffmann and colleagues described RITA, realtime RT-PCR assay for wide spectrum of influenza A viruses, including H2 strains [13]. Yet, we had no opportunity for experimental comparison of RITA and our protocol for influenza A(H2) detection.

In this study, two sets of primers and TaqMan probes were designed based on the conserved regions of the HA gene of influenza A(H2) viruses. Conserved regions were identified by analysis of Shannon entropy distribution. Developed assays successfully passed in silico evaluation and were selected for further experimental evaluation. rRT-PCR efficiency was experimentally estimated to be around 100% fulfilling MIQE criteria for PCR assays [23]. Described primer/probe H2-1 and H2-2 sets showed different specificity on different influenza strains. H2-2 set demonstrated false positive results on some seasonal influenza A(H1N1) strains and pooled influenza positive control (PIPC). Thus H2-2 was considered to be inappropriate for use and excluded from further experimental evaluation.

H2-1 rRT-PCR assay showed moderate sensitivity. Plasmid DNA template detection limit of the assay was 50 copies per 25 µl reaction. Limit of detection tested on serial dilutions of infectious virus was $10^{3.2}$ EID₅₀/ml (3.2 (CI95%: 3.07–3.48) lg EID₅₀/ml). Obtained LOD is higher, than those for the real-time quantitative RT-PCR targeting the M gene, which are usually used as the “gold standard (e.g. $10^{0.3}$ TCID₅₀/ml for H7N9 assay [24], $10^{1.3}$ EID₅₀/ml for CDC Influenza 2009 A(H1N1)pdm Real-Time RT-PCR Panel [25]), but we consider it acceptable for qualitative RT-PCR testing. Using artificial human specimens we demonstrated that developed H2-1 assay is suitable for use on human specimens containing diverse interfering substances.

In summary, a realtime RT-PCR assay (H2-1) for detection of the influenza A(H2) viruses was designed, and was shown to have adequate sensitivity and specificity. Availability of realtime RT-PCR assays for potentially pandemic influenza viruses is important for pandemic preparedness, making easier early identification of pandemic strain.

Author contributions

All authors have contributed to, seen and approved the manuscript. Andrey Komissarov performed influenza A(H2N2) sequence analysis, designed primers and probes, performed RT-PCR testing and wrote the manuscript, Artem Fadeev wrote Python script for Shannon entropy calculation, performed RT-PCR testing, determined virus titers and participated in the writing of the manuscript, Anna Kosheleva performed capillary sequencing, RT-PCR testing and participated in the writing of the manuscript, Kseniya Sintsova performed RT-PCR testing, figure preparation, statistics and participated in discussions, Mikhail Grudin participated in the writing of the manuscript, provided his comments and participated in discussions.

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pCI-neo Expression Vector Containing H2 HA Gene from Influenza A/Japan/305/1957 (H2N2), FR-576, was obtained through the Influenza Reagent Resource, Influenza Division, WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mcp.2017.06.005>.

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