Application of three duplex real-time PCR assays for simultaneous detection of human seasonal and avian influenza viruses

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Abstract This study was performed to develop real-time PCR (qPCR) for detection of human seasonal and avian influenza viruses in duplex format. First duplex qPCR detects haemagglutinin (HA) gene of influenza virus A(H1N1)pdm09 and HA gene of influenza virus A(H3N2), the second reaction detects neuraminidase (NA) gene of influenza virus A(H3N2) and NA gene of influenza virus A(H1N1)pdm09 and A(H5N1), and the third reaction detects HA gene of influenza A(H5N1) and nonstructural protein gene of influenza B virus. Primers and probes were designed using multiple alignments of target gene sequences of different reference strains. Assays were optimised for identical thermocycling conditions. Their specificity was confirmed by conventional PCR and monoplex qPCR with nucleic acids isolated from different influenza viruses and other respiratory pathogens. Plasmid constructs with a fragment of specific gene were used to assess sensitivity of the assay. The limit of detection ranged from 27 to 96 cDNA copies/reaction. Clinical specimens (n = 107) have been tested using new assays, immunofluorescence and monoplex qRT-PCR. It has been shown that developed assays have been capable of rapid and accurate simultaneous detection and differentiation of influenza viruses. They are more sensitive than immunofluorescence and at least as sensitive as monoplex qRT-PCR.

Introduction

Since 2009, influenza viruses of the A(H1N1)pdm09 and A(H3N2) subtypes as well as influenza B viruses have been responsible for human influenza diseases in the northern hemisphere [11, 12, 33, 35, 37]. Nevertheless, infections with a highly pathogenic avian influenza virus A(H5N1) have been observed in the human population. According to data provided by the World Health Organization (WHO), as of August 10, 2012, a total of 608 human infections caused by A(H5N1) were laboratory confirmed, and 359 fatal cases (59.0 %) were reported since 2003 [36]. Thus, despite the fact that the end of the pandemic caused by A(H1N1)pdm09 was announced in August 2010, there is still a potential threat of another influenza pandemic caused by HPAI A(H5N1) virus [32]. Certainly, the high level of changeability of influenza viruses together with an animal reservoir for these pathogens makes it possible that a completely new virus variant with pandemic potential will emerge [6, 21, 30]. Therefore, high-quality surveillance together with the availability of rapid and accurate diagnostic methods is a big challenge in the case of influenza virus infections. Subtyping of influenza viruses is necessary for surveillance and usually is less important for the diagnosis of an individual patient’s illness, since a clinical
Materials and methods

Reference strains and specimens

The reference strains of influenza viruses A(H3N2) (A/Moscow/10/1999; A/Christchurch/28/2003; A/California/7/2004; A/Wisconsin/67/2005; A/Brisbane/10/2007; A/Perth/16/2009), A(H1N1)pdm09 (A/California/7/2009, A/Denmark/528/2009), A(H1N1) (A/Salomon Islands/3/2006, A/Brisbane/59/2007, A/Fukushima/141/2006) and B (B/Shanghai/361/2002, B/Malaysia/2506/2004, B/Florida/7/2004, B/Egypt/144/2005, B/Florida/4/2006; B/Brisbane/60/2008) used in the present study were kindly provided by the World Health Organization, Collaborating Centre for Reference and Research on Influenza (London, UK). Viruses were propagated in the allantoic cavities of 11-day-old chicken embryoenated eggs. Then, collected fluids were titrated using a haemagglutination test performed with turkey red blood cells and were stored at −70 °C [20]. Inactivated A(H5N1) strains A/Duck/Vietnam/TG24-01/2005 and A/Whooper Swan Germany/R65-2/2006 were kindly provided by the Robert Koch Institut, Nationales Referenzzentrum für Influenza (Berlin, Germany). Other respiratory viruses: human respiratory syncytial virus A and B (RSV-A, -B), human parainfluenza virus 1, 2 and 3 (PIV-1, -2, -3), human adenovirus (hAdV); human metapneumovirus (hMPV), human rhinovirus (hRV), and human coronavirus (hCoV) HKU1 and NL63, were kindly provided by the Department of Virology, Erasmus Medical Center (Rotterdam, The Netherlands). The reference strains of Streptococcus pneumoniae (ATCC 6301), Klebsiella pneumoniae (ATCC 4211) and Haemophilus influenzae (ATCC 9006) were provided by the Chair and Department of Medical Microbiology, Medical University of Warsaw, Poland.

The clinical usefulness of the developed assays was confirmed by testing RNA obtained from 57 samples provided by the quality control panels WHO External Quality Assessment Programme (WHO EQAP) and Quality Control for Molecular Diagnostics (QCMD; http://www.qcmd.org) (A(H1N1)pdm09, n = 10; A(H3N2), n = 10; A(H5N1), n = 22; influenza B virus, n = 7; and 8 samples negative for influenza A and B virus). The A(H5N1) RNA samples from EQAP panels belonged to different clades: 1, 2.1, 2.2, 2.3.2., 2.3.4.

A comparison of different laboratory techniques with the newly developed assays was done using 107 respiratory specimens (combined nose and throat swabs) collected from patients with flu symptoms in Poland during the influenza epidemic seasons 2008/2009 (n = 27), 2009/2010 (n = 55) and 2010/2011 (n = 25).

Primer and probe design

Primers and fluorescent probes, based on TaqMan chemistry, were designed to correspond to specific highly conserved regions of the influenza virus A haemagglutinin (H1)pdm09 (but not seasonal influenza A(H1N1) viruses), the H3 and H5 genes, the influenza virus A neuraminidase N1 and N2 genes, and the influenza B virus (both Yamagata and Victoria lineage) nonstructural protein (NS) gene. The most specific and conserved target regions were identified following multiple alignments of the nucleotide sequences of the respective HA/NA/NS genes of different influenza virus strains available from the Influenza Sequence Database (ISD) [19] and GenBank database (National Center of Biotechnology Information). Primer and probe sets were
Table 1 Sequences of primers and probes designed and selected for use in the qPCR assays

| Name      | Oligonucleotide sequence (5’–3’)                                      | Fluorophore (5’/3’) | Amplicon length (nt) | Target (gene) | Nucleotide positions | GenBank accession no. of reference sequence |
|-----------|-----------------------------------------------------------------------|---------------------|----------------------|---------------|----------------------|---------------------------------------------|
| H1v_F     | GTATTATCATTGACAGATACACAGGTCC                                         |                     |                      |               | 842-868              |                                             |
| H1v_R     | GACATTTTCCAATGTGATCGG                                                |                     |                      | HA           | 961-940              | FJ981613                                    |
| H1v_P     | AACACCAGGCTCCCATTTTCGAG                                              | JOE/BHQ1            | 119                  | HA           | 910-932              | (H1N1)pdm09                                 |
| H3_F      | TTGATCAACAGCAGGGAATCTA                                               |                     |                      | HA           | 778-801              |                                             |
| H3_R      | TGCAATTTGCCATATTGCATTGGCC                                            |                     |                      | HA           | 892-821              | GQ293081                                    |
| H3_P      | AGCTCAATAATGAGATCAGATGACC                                            | CFR610/BHQ2         |                      | H3N2         | 841-866              |                                             |
| H5_F      | TTTTATGCCGAAATATGCATACAA                                             |                     |                      | HA           | 786-811              |                                             |
| H5_R      | GGATGGCATACTAGATTGTATCG                                              |                     |                      | HA           | 922-899              | DQ464354                                    |
| H5_P      | ACTGCAACAGGACAGGTCAAACCTCC                                           | JOE/BHQ1            | 136                  | HA           | 865-889              | H5N1                                       |
| B_F       | GATCCCTAATCTCTGGCAG                                                  |                     |                      | NS           | 717-737              |                                             |
| B_R       | CTTCTTCGTTGATAGATAATGTGG                                             |                     |                      | NA           | 837-817              | JN992795                                    |
| B_P       | ACATCTCAACGTTAAGGAACAGCT                                             | CFR610/BHQ2         | 121                  | NA           | B                    |                                             |
| N1_F      | TAAAACCTGCTCTGGGTT                                                   |                     |                      | NA           | 1253-1272            |                                             |
| N1_R      | GCAAAAGCCACAGTGTG                                                    |                     |                      | NA           | 1367-1351            | FJ984386                                    |
| N1_P      | GCAGCATATCTTCTGTGTTG                                                 | CFR610/BHQ2         | 142                  | NA           | 1321-1342            | (H1N1)pdm09                                |
| N1(H5)_F  | AGTTGGTGGTGAATGAGATGACC                                              |                     |                      | NA           | 505-526              |                                             |
| N1(H5)_R  | TTAACATGCACATCAGACT                                                  |                     |                      | NA           | 643-623              | DQ464355                                    |
| N1(H5)_P  | GCATAAAACAGACACACATGACAGATGAGTGAGAAGAAGAAGAAGTC                     | CFR610/BHQ2         | 138                  | NA           | 587-601              | H5N1                                       |
| N2_F      | CCTTGGATGATGGAATGACG                                                 |                     |                      | NA           | 1058-1078            |                                             |
| N2_R      | CTGTCAACCTTGACCTGCT                                                  |                     |                      | NA           | 1199-1180            | CY081429                                    |
| N2_P      | AAAAGTCACTTTAGAGGTCAGTCCAANC                                       | JOE/BHQ1            | 141                  | NA           | 1131-1155            | H3N2                                       |

HA = haemagglutinin; NA = neuraminidase; NS = nonstructural protein; F = forward primer; R = reverse primer; P = probe; r = A, G; m = A, C; CFR610 = California Fluor Red 610, BHQ = Black Hole Quencher
designed according to all basic rules [18, 23], using PrimerQuestSM (Integrated DNA Technologies, Inc. http://eu.idtdna.com/Scitools/Applications/Primerquest/Advanced.aspx) and LightCycler Probe Design2 software (Roche Diagnostics). The reference sequences were as follows: influenza A(H1N1)pdm09 virus, accession no. FJ981613 (HA) and FJ984386 (NA); A(H3N2) virus, accession no. GQ293081 (HA) and CY081429 (NA); A(H5N1) virus, accession no. DQ464354 (HA) and DQ464355 (NA); influenza B virus, accession no. JN992795 (NS).

Primer and probe sequences were analysed for their G+C content and formation of dimers, hairpins and secondary structures using OligoAnalyzer 3.1 (Integrated DNA Technologies, Inc; http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). The specificity of each primer and probe set was measured using BLAST against the entire human genome and influenza viruses other than targeted ones to prevent the amplification of non-specific, sequence-related secondary products. All oligonucleotides were synthesised by Genomed S.A. (Warsaw, Poland). The sequences of the primers and probes are shown in Table 1.

### Nucleic acid extraction

A QIAamp Viral RNA Mini Kit (QIAGEN, Germany) was used to purify total RNA from 140 μl of allantoic fluid (reference strain) or clinical specimens. DNA was isolated from 200 μl of bacterial samples using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics). All isolations were performed according to the manufacturer’s instructions, and extracted nucleic acids were eluted in a final volume of 50 μl of elution buffer. Isolated nucleic acids were stored at −70 °C.

### Reverse transcription

The viral RNA was reverse-transcribed to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) with random hexamer primers and 5.0 μl RNA template according to the manufacturer’s instructions. Negative controls were included in each run. The cDNA samples that were obtained were stored at −70 °C until further analysis.

### Conventional PCR

The reaction mixture contained Hot Start PCR Buffer (Thermo-Scientific Fermentas), 2.5 μl dNTP mix (0.2 mM each), 2.0 mM MgCl2, 20 μM each primer, 1.0 U Maxima Hot Start Taq DNA polymerase (Thermo-Scientific Fermentas), 2.5 μl of cDNA (influenza B, A(H1N1)pdm09, A(H3N2) or A(H5N1)) and sterile DNase/RNase-free water to a final volume of 25.0 μl. Amplification was performed in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems Inc., USA) as follows: a single cycle of initial denaturation/enzyme activation for 4 min at 95 °C, then 45 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 15 s and extension at 72 °C for 20 s. After the last cycle, the reaction was completed by a final extension at 72 °C for 5 min. PCR products in a total volume of 20 μl were separated in a 2.0 % agarose gel stained with GelRed™ solution in 1× TAE buffer. A GeneRuler 100 bp DNA Ladder was used for estimating the molecular size of the bands obtained (Thermo-Scientific Fermentas).

### Real-time PCR

In order to optimise the reactions, the monoplex and duplex reactions were carried out with 1.0, 1.5 or 2.0 μM primers and 100, 150 or 200 nM probes. The assays were performed in a two-step reaction using a LightCycler® TaqMan® Master Kit (Roche Diagnostics) and 5.0 μl of cDNA.

Assays were optimised for using one identical set of thermocycling conditions. Fluorescence levels were measured at the wavelengths 560 nm and 610 nm at the end of the elongation step of each cycle. All qPCR reactions and data analysis were performed using a LightCycler® 2.0 instrument (Roche Diagnostics). Colour compensation software was used in the analysis according to the manual provided by Roche Diagnostics. The crossing point (Cp) value was calculated by determining the point (PCR cycle) at which the fluorescence intensity rises above background (detection limit). Positive and negative controls were included in each run.

### Table 2 Characteristic of HA, NA and NS gene fragments included in plasmid constructs

| Nucleotide positions | GenBank accession no. | Strain Origin | Origin |
|----------------------|-----------------------|---------------|--------|
| 706-1090             | FJ981613              | A/California/07/2009 (H1N1)09pdm | Segment 4, H1 |
| 1026-1410            | FJ984386              | A/Perth/16/2009 (H3N2) | Segment 4, H3 |
| 666-1050             | GQ293081              | A/Perth/16/2009 (H3N2) | Segment 4, H3 |
| 936-1320             | CY081429              | A/Sweden/Germany/R-65/2006 (H5N1) | Segment 4, H5 |
| 673-1057             | DQ464354              | A/Sweden/Germany/R-65/2006 (H5N1) | Segment 6, N1 |
| 376-760              | DQ464355              | A/Sweden/Germany/R-65/2006 (H5N1) | Segment 7, N1 |
| 593-977              | JN992795              | B/Florida/02/2010 | Segment 8, NS |
In order to assess the sensitivity of the assays, seven plasmid constructs were constructed by cloning a fragment of the HA or NA gene (385 bp) of influenza A(H1N1)pdm09, A(H5N1), and A(H5N1) virus and the NS protein gene of influenza B virus into Smal-digested pBluescript II SK(−) (Table 2) (Epoch LifeScience, Missouri City, USA). Plasmid DNA was sequenced to confirm correct insertion into the vector. DH5α electrocompetent E. coli cells were transformed with 100–150 ng of each construct using a MicroPulser™ (Bio-Rad) according to the standard protocol. Plasmid DNA was purified using a Plasmid Midi AX Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer’s instructions. The concentration of recombinant plasmid DNA was determined spectrophotometrically by measuring the absorbance of UV light at 260 nm and 280 nm. Plasmid DNA was cut with the restriction enzyme PdiI (Thermo-Scientific Fermentas) and serially diluted in sterile, DNase/RNase-free water. The limit of detection (LOD) of each assay was determined by analysis of tenfold serial dilutions of plasmid DNA in the range of 10^2–10^4 copies. Each dilution was prepared and analyzed in six independent replicates. Probit analysis was used to calculate the LOD concentration [4].

Comparison with immunofluorescence assay and monoplex RT-PCR

A total of 107 respiratory specimens were tested using the new duplex qPCR assays in comparison with the following standard rapid laboratory methods: immunofluorescence assay (IFA) for influenza A and B viruses and monoplex qPCR for influenza A(H1N1)pdm09 and A(H3N2) viruses and influenza B virus. The IFA was performed using the commercial IMAGEN™ Influenza A and B Kit (Oxoid) according to the manufacturer’s instructions. Monoplex qPCR was performed with primers/probe sets from the Centers for Disease Control and Prevention (CDC) RT-PCR Protocol for detection of influenza A(H1N1)pdm09 (WHO, 2009) and influenza A(H3N2) and B viruses (available as part of a material transfer agreement with CDC). Amplification was carried out in a 25.0-µl reaction volume using a SuperScript® III Platinum® One-Step qRT-PCR Kit (Invitrogen Life Technologies) and 5.0 µl of extracted RNA template. The conventional PCR with primers specific for β-actin was performed for all negative samples.

Results

The purpose of the present study was the development of qualitative real-time PCR assays for the detection and differentiation of seasonal and avian influenza viruses.

Assay optimisation

Real-time PCR assays were first performed as monospecific assays with cDNA from targeted viruses and optimised to increase the sensitivity and efficiency of amplification. The monospecific assays were then combined in three duplex reactions and then optimised. The concentrations of primers and probes that gave the best results were as follows: 1.0 µM for influenza virus H3-specific primers and 1.5 µM for influenza virus H1pdm09-, H5-, N1-, N2- and B-specific primers, and 150 nM for each of the probes in a total volume of 20 µl of the reaction mixture. A duplex real-time PCR system was used to identify (i) the (H1)pdm09 and H3 subtypes, (ii) the N2 and N1 subtypes, and (iii) the H5 subtype and the B type. Assays were performed in a two-step reaction and optimised for using the same thermocycling conditions. The best amplification results were obtained with activation of thermostable hot-start DNA polymerase for 10 min at 95 °C, followed by 40 cycles comprising denaturation (10 s at 95 °C), annealing (20 s at 60 °C) and strand elongation (5 s at 72 °C). At the end of each strand-elongation step, a single measurement of fluorescence was performed.

Specificity of RT-PCR and duplex qPCR

The selected primers and probes showed no nonspecific homologies in a nucleotide BLAST search including human genomic and influenza viruses other than the targeted ones. To confirm their specificity experimentally, each assay was performed with nucleic acids isolated from the reference strains of different influenza viruses and a panel of common human respiratory pathogens as listed in Materials and methods. All primer/probe sets detected only RNA (cDNA) from the corresponding virus in the conventional PCR as well as in the newly developed qPCR assays (in monoplex and duplex format). No nonspecific signals were observed, and there was no cross-reaction with other common viral or bacterial respiratory pathogens (Fig. 1A, B, C). Duplex qPCR with combined templates from different influenza viruses gave specific and proper results without cross-talk of fluorescence from the 560 nm and 610 channels when the colour compensation software was applied.

Clinical performance

In the present study, 57 samples from control panels that were previously diagnosed as positive for influenza A(H1N1)pdm09, A(H3N2), A(H5N1), influenza B or negative for influenza (monoplex one-step qPCR or conventional RT-PCR; data not shown) were used to evaluate
the clinical usefulness of the newly developed assays. None of the tested samples showed any false-positive results or nonspecific signals (Fig. 2A, B, C). All positive specimens were found to be positive when tested using our duplex qPCR assays. Despite the high degree of genetic variability and co-circulation of different clades of A(H5N1) subtypes, our assay identified the HA genes of all of the currently circulating clades examined. In eight negative specimens, influenza virus A(H1N1)pdm09, A(H3N2), A(H5N1) and B were not detected, and moreover, no amplified product was visible after agarose gel electrophoresis of qPCR negative samples. These results confirmed, in 100 % of the samples, both the specificity and the clinical usefulness of these assays.

**Sensitivity of duplex qPCR**

In the qPCR assay, all serial dilutions of the template, containing $10^4$ to $10^5$ plasmid DNA copies were successfully detected. The results of LOD in the duplex qPCR assays, expressed as DNA copies per reaction tube, are shown in Table 3.

**Comparison of duplex qPCR with standard methods**

A total of 107 respiratory specimens were tested using the newly developed assays and the standard rapid laboratory methods IF and monoplex qPCR, as recommended by the CDC. All results of monoplex qPCR were consistent with those obtained by duplex qPCR, i.e., 60 samples were identified as influenza A(H1N1)pdm09, 17 samples as influenza A(H3N2), 18 samples as influenza B and 12 samples were negative for influenza virus. Neither false positive nor false negative results were observed. The results of IFA were consistent with both qPCR assays for 96 specimens (73 influenza A positive, 14 influenza B positive and 9 influenza A and B negative). Eight specimens that were negative for influenza A and B virus by IF assay, were subsequently determined to be positive in both qPCR assays for influenza A(H1N1)pdm09 (n = 3), A(H3N2) (n = 1) and B (n = 4). Three specimens that were positive for influenza A virus in the IF assay were negative in both qPCR assays. Positive results of amplification of the β-actin gene were obtained for all samples.

**Discussion**

Early detection and virological surveillance of influenza virus strains circulating regionally and globally is a key element in both updating the influenza vaccine composition and epidemic/pandemic prevention and control [29, 34]. The co-circulation of multiple types and subtypes of seasonal influenza viruses increases the difficulty of virus identification and diagnosis. Molecular methods based on PCR allow rapid, accurate, sensitive and direct detection of influenza viruses, including subtyping of influenza virus A, and have become a valuable tool in a routine diagnostics as well as surveillance. However, the high level of genetic variability of influenza viruses, especially among viruses of type A, makes the development of specific molecular assays difficult. Simultaneously, this changeability requires diagnostic methods to be constantly monitored and updated in the event of the emergence of a new genetically distinct strain. Many multiplex qPCR assays for detection of influenza viruses have been developed [2, 5, 7–9, 14, 27, 28]. Nevertheless, to our knowledge, there is only one paper describing multiplex assays for the detection of each of the following influenza viruses: type B, subtypes A(H3N2), A(H5N1) and A(H1N1) but without differentiation between A(H1N1)pdm09 and seasonal A(H1N1) [24]. Thus, three duplex qPCR assays designed in this study for concurrent detection of influenza B, A(H3N2), A(H1N1)pdm09 and A(H5N1) viruses offer a valuable strategic diagnostics approach and could be routinely adapted for the identification of not only seasonal influenza viruses circulating in the human population but also the unusual H5N1 subtype of avian origin.

Genes that encode internal virus proteins such as the non-structural NS protein are highly conserved among different influenza viruses and thus are useful targets for the universal detection of virus type [34]. Since all influenza virus subtypes are defined on the basis of differences in their surface glycoproteins, haemagglutinin and neuraminidase gene targets are the most effective and
In the present study, the corresponding genes have been also shown to be useful for differentiation of specific types and subtypes. However, because of the large variability among A(H5N1) strains, two primer/probe sets were selected for the efficient detection of N1. Both probes were labelled with the same fluorophores because the duplex reaction was designed for identification of N1 without differentiation between A(H1N1)pdm09 and A(H5N1).

In multiplex qPCR, amplicons are discriminated by using distinct fluorogenic probes for each nucleic acid target or, alternatively, melting curves when non-specific DNA-intercalating fluorophores are used as the detection method [18]. The limited number of fluorophores, which show significant overlap in their emission spectra, as well as the finite number of emission channels in a qPCR instrument, limits the number of targets that can be detected in a single reaction by multiplexing [15, 18]. In the present study, a LightCycler® 2.0 system with an LED light source was used. A duplex format of the reaction was based on hydrolysis of probes labelled with JOE reporter dye and the quencher BHQ-1 or California Fluor Red 610 and the quencher BHQ-2. Despite the two different emission maxima of the fluorophores and measurement of fluorescence in two different detection channels (560 nm for JOE and 610 nm for California Fluor Red 610), the use of color compensation software was necessary due to overlapping of fluorescence. According to literature data [15] as well as our results (data not shown), the LightCycler® 2.0 is an appropriate and well-working platform to detect up to two different fluorophores in a single reaction with TaqMan probes. Designed primer and probe sets labelled in the proposed format can be differently combined and, after validation, used in other duplex formats: (H1)pdm09 with N1, H3 with N2, and H5 with N1. Then, a complete identification of a specific subtype, including haemagglutinin and neuraminidase genes, is possible.

In clinical evaluation based on respiratory specimens collected from patients with influenza symptoms in three epidemic seasons, the concordance rate between the results of the newly developed and validated duplex qPCR and monoplex qPCR assays recommended by CDC was 100.0 %. Nevertheless, comparison with the IF assay showed an advantage of molecular methods. The discrepancy results between qPCR and IFA comprised 10.3 % (11/107) of the total. The newly developed assays resulted in eight additional positives that were missed by the routine IF method. Moreover, three specimens that were positive for influenza A virus in IFA were actually negative, as each qPCR assay and also virus isolation (data not shown) gave negative results. These data are consistent with numerous reports showing that PCR-based methods are more sensitive than IFA for the detection of viral infections [3, 10, 16, 17, 22].

Differences in the detection of viruses by various tests can be explained at least partially by the different targets used for each method. The sensitivity of IFA is significantly influenced by the quality of the specimen, since virus-infected cells are very labile and may be easily damaged by inappropriate handling or storage of clinical specimens before processing. Moreover, the analysis and interpretation of results obtained by IFA are often subjective, depending on the experience of the investigator. Molecular methods allow the detection of nucleic acids from viable as well as non-viable viruses, even when the viral copy number is low, and thus exhibit the highest sensitivity. Although PCR methods may be affected by PCR inhibitors, the occurrence of inhibition of amplification in respiratory specimens is rare. Among 545 respiratory specimens tested by Stauffer et al. [25], only 12 (2.2 %) were inhibited in PCR. In addition, RNA/DNA-degrading enzymes present in the specimens, as well as reagent degradation, may reduce the sensitivity of PCR.
surveillance and early diagnosis. The inclusion of highly conserved regions of circulating influenza viruses than detection of only one gene [31]. The inclusion of highly conserved regions of two different target genes (HA and NA) in the assay described here definitely increases the possibility of detection of a new influenza A strain and significantly decreases the risk that, in the event of a mutation, detection of a specific virus will fail. In the present study, the selection of the target sequences used for the qPCR assays did not contribute to any false negative results.

In conclusion, the three newly developed and validated duplex qPCR assays are capable of rapid and accurate simultaneous detection of influenza viruses, allowing influenza B virus and all currently circulating strains of human seasonal influenza A viruses (H1N1pdm09 and H3N2) as well as avian influenza A(H5N1) to be differented. All assays can be run under the same thermal conditions, providing a valuable method for both influenza surveillance and early diagnosis.

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