Development and Evaluation of Multiplex Real-time RT-PCR Assays for Seasonal, Pandemic A/H1pdm09 and Avian A/H5 Influenza Viruses Detection

Jang-Hoon Choi, Mi-Seon Kim, Joo-Yeon Lee, Nam-Joo Lee, Donghyok Kwon, Min Gu Kang, and Chun Kang*

Division of Influenza Virus, Center for Infectious Disease, Korea National Institute of Health, Korea Center for Disease Control and Prevention, Osong 363-951, Republic of Korea

(Received August 29, 2012 / Accepted January 2, 2013)

Since the pandemic influenza A (H1N1) 2009 ((H1N1)pdm09) virus spread all over the world, the (H1N1)pdm09 virus has been circulating with seasonal influenza viruses. We developed rapid and sensitive one-step multiplex real-time RT-PCR assays (rRT-PCR) for simultaneous detection of influenza viruses currently circulating in humans, and the avian A/H5 virus. The detection limit of each assay was 4.8 to 1 copies per reaction and no cross-reactivity with other major respiratory pathogens was found. Analytical positive predictive value (PPV), negative predictive value (NPV) sensitivity and specificity were 100%, 94.1%, 93.7% and 100%, respectively. Clinical evaluation revealed that 1,976 (16.5%) of 11,963 throat swabs from patients with respiratory symptoms were confirmed as 1,651 (83.6%) A/H1pdm09, 308 (15.6%) A/H3 and 17 (0.8%) B virus during the 2010-2011 influenza season. Collectively, the multiplex rRT-PCR assays described here provide a practical tool for reliable implementation of influenza surveillance and diagnosis.

Keywords: influenza, real-time RT-PCR, surveillance, diagnosis

Introduction

Influenza viruses are the major causative agents of human acute respiratory disease worldwide, with potentially severe consequences for human health and the economy. The most common symptoms of the disease are chills, fever, sore throat, muscle pains, headache, coughing, fatigue, and general discomfort (Eccles, 2005). Although the incidence of influenza varies widely from year to year, approximately 36,000 deaths and more than 200,000 hospitalizations are directly associated with influenza every year in the United States (Thompson, et al., 2003).

A novel strain of the H1N1 virus that originated in swine was identified by the Centers for Disease Control and Prevention (CDC) in April 2009, becoming a pandemic strain and spreading across the world (CDC, 2009; Dawood et al., 2009). In Korea, the first confirmed case was identified on May 2nd - a person who had travelled to Mexico following the outbreak there. Since then, the pandemic virus spread rapidly until early 2010 and by January 2010 there were 16,447 cases that had been confirmed by laboratories in public health institutes. At the beginning of the pandemic A (H1N1) 2009 ((H1N1)pdm09) virus outbreak, there was no specific molecular detection system until the World Health Organization (WHO) recommended protocol (CDC rRT-PCR swine Flu panel) became available (WHO, 30 April 2009). In-house conventional RT-PCR based on A/California/04/2009 virus genome sequences was the only applicable method in the Korea National Influenza Center (NIC). However, additional sequence analysis was needed for confirmation at that time. In May 2009, the Korea NIC introduced the CDC real-time RT-PCR (rRT-PCR) swine Flu panel with minor modifications and used it for clinical diagnosis until the end of the pandemic. Thereafter several multiplex rRT-PCR for large scale influenza surveillance and diagnosis have been developed by other groups (Chidlow et al., 2010; Huber et al., 2011).

In Korea, Seasonal H1N1 and H3N2 viruses dominantly circulated with distinct seasonality until the emergence of the (H1N1)pdm09 virus. Moreover, the highly pathogenic avian influenza (HPAI) H5N1 virus has prevailed continuously in domestic poultry farms in Korea since 2003 (Lee et al., 2005, 2008). Therefore, an adequate detection system targeting currently circulating human strains, as well as HPAI H5N1, is also required.

This article describes the development and evaluation of rapid and sensitive one-step multiplex real-time assays that are capable of detecting influenza circulating in humans for large scale surveillance and diagnosis.

Materials and Methods

Design of primers and probes

The nucleotide sequences of the matrix (M), hemagglutinin (HA), and nucleoprotein (NP) genes of influenza viruses were obtained from the GenBank database. The M gene, conserved for influenza A, was selected for the detection of type A influenza virus. The M gene-specific primer and probe set was designed based on sequence comparison among
Real-time RT-PCR for influenza viruses detection

Table 1. Primer and probe sets of multiplex real-time RT-PCR assays

| Multiplex set     | Target gene           | Primer/probe       | Sequence (5’→3’)                   | Product size (bp) |
|-------------------|-----------------------|--------------------|------------------------------------|-------------------|
| 1                 | Influenza type A Matrix | Forward            | AAT CCT GTC ACC TCT GAC TAA GG     | 99                |
|                   |                       | Reverse            | CAT TYT GGA CAA ARK GTC TAC G      |                   |
|                   |                       | Probe              | **FAM-TGC AGT CCT CGC TCA C-MGBNFQ**|                   |
|                   | Influenza type B Nucleoprotein | Forward    | GAA TGC TGT CAA TGA ATA TGG AGG G  | 77                |
|                   |                       | Reverse            | CAT TGA TTC ATT CAT CCT GAG TAG AT  |                   |
|                   |                       | Probe              | **VIC-TCC TTT GAC ATC TGC AT-MGBNFQ**|                   |
| 2                 | A/H1pdm09 Hemagglutinin | Forward            | GCA CTC ATT CTG AGG GGA TCA G      | 67                |
|                   |                       | Reverse            | TGC AAG CCC ATA CAC ACA AGC        |                   |
|                   |                       | Probe              | **FAM-CAC ATA AAT CCT GCC TGC-MGBNFQ**|                   |
| 3                 | Avian A/H5 Hemagglutinin | Forward            | GGT AAC GTG TGT TTC GAG TTC TAT CA | 89                |
|                   |                       | Reverse            | AAT ACT GGG GGT AGT CAT ACG TTC C  |                   |
|                   |                       | Probe              | **NED-ATG TGA TAA TGA ATG TAT GGA AAG T-MGBNFQ**| |
|                   | GAPDH<sup>a</sup>     | Forward            | AGA TTT GGA CCT GCG AGC G          | 65                |
|                   |                       | Reverse            | GAG CCG CTG TCT CCA GTA            |                   |
|                   |                       | Probe              | **Cy5-TTC TGA CCT GAA GCC TGC CG-BHQ**|                   |
| 4                 | Seasonal A/H1 Hemagglutinin | Forward            | CCC CAA GAC GAG TTC ATG GC         | 150               |
|                   |                       | Reverse            | AGC ATG AGG ACA TGC TGC C          |                   |
|                   |                       | Probe              | **FAM-CAT GAC TCG AAC AATG GGA-MGBNFQ**|                   |
| 5                 | Seasonal A/H3 Hemagglutinin | Forward            | TAG AAA ATG GAT GGG AGG GAA TG     | 102               |
|                   |                       | Reverse            | CTG CTT GAG TGG TTT TAA GAT CTC G |                   |
|                   |                       | Probe              | **VIC-TGG TAC GGT TTCA GCC AT-MGBNFQ**|                   |

<sup>a</sup>Glyceraldehyde 3-phosphate dehydrogenase : internal control.

Different subtypes of over 200 strains of influenza A virus from human, swine and avian sources. Each subtype specific HA primer and probe set was designed based on the alignment of over 100 HA sequences of representative viruses from Asia, America and Europe. The influenza B primer and probe set was designed for the conserved NP gene between Victoria and Yamagata lineages. All primer and probe sets were designed by using PrimerExpress (Version 5.0; Applied Biosystems, USA) software. Selected primer and probe sequences were compared with sequences submitted to the GenBank nucleotide database using a standard nucleotide-nucleotide comparison tool: BLASTN (Version 2.2.1; NCBI, MD, http://www.ncbi.nlm.nih.gov/). The probes were labeled with minor groove binding no fluorescence quencher (MGBNFQ) or black hole quencher (BHQ) at the 3’ end, and four different fluorescent reporter dyes (FAM, VIC, NED and CY5) at the 5’ end, so that the multiple genes of an influenza virus could be detected simultaneously in a single tube (Table 1).

Table 2. List of human respiratory pathogens used for specificity test

| Viruses and bacteria<sup>b</sup> | Source<sup>b</sup> | Titer                      |
|----------------------------------|--------------------|----------------------------|
| RNA viruses                      |                    |                            |
| PIV1/2/3                         | ATCC VR-26         | > 5×10<sup>5</sup> TCID<sub>50</sub>/ml |
| RSV                              | ATCC VR-43         | 4×10<sup>5</sup> TCID<sub>50</sub>/ml |
| HCoV 229E                        | ATCC VR-740        | 3×10<sup>5</sup> TCID<sub>50</sub>/ml |
| HCoV OC43                        | ATCC VR-1558       | 4×10<sup>5</sup> TCID<sub>50</sub>/ml |
| Rhinovirus 13                     | ATCC VR-1123       | 1×10<sup>6</sup> TCID<sub>50</sub>/ml |
| Enterovirus 68                    | ATCC VR-561        | 5.2×10<sup>6</sup> TCID<sub>50</sub>/ml |
| DNA viruses                      |                    |                            |
| HBoV                             | ATCC VR-540        | 1×10<sup>6</sup> copies/ml |
| Adenovirus 3                     | ATCC VR-1480       | 5×10<sup>5</sup> pfu/ml    |
| HSV-2                            | ATCC VR-1503       | 2.5×10<sup>6</sup> TCID<sub>50</sub>/ml |
| HHV-6                            | ATCC VR-1480       | 3×10<sup>5</sup> TCID<sub>50</sub>/ml |
| VZV                              | ATCC VR-1503       | 4.5×10<sup>5</sup> TCID<sub>50</sub>/ml |
| Bacteria                         |                    |                            |
| *Haemophilus influenzae*         |                    | 2×10<sup>5</sup> cell/ml  |
| *Legionella pneumophila*         |                    | 3×10<sup>5</sup> cell/ml  |
| *Streptococcus pneumoniae*       |                    | 1.5×10<sup>5</sup> cell/ml|
| *Mycoplasma pneumoniae*          |                    | 3×10<sup>5</sup> cell/ml  |
| *Chlamydia pneumoniae*           |                    | 1×10<sup>5</sup> cell/ml  |

<sup>b</sup>HIV-2, Herpes simplex virus 2; HHV-6, Human herpesvirus 6; VZV, Varicella-zoster virus; PIV1/2/3, Parainfluenza virus 1/2/3; RSV, Respiratory syncytial virus; HCoV, Human coronavirus; HBoV, Human bocavirus
<sup>a</sup>These bacteria were provided by Division of Bacterial Respiratory Infections, Center for Infectious Diseases, Korea National Institute of Health.
<sup>b</sup>If not specified, strains are clinical isolates.
Viral RNA extraction

Viral RNA was extracted from 140 μl of viral transport medium (VTM) or tissue culture fluid of each sample using a QIAamp viral RNA mini kit (Qiagen, UK), according to the manufacturer’s instructions.

Multiplex rRT-PCR

A one-step multiplex rRT-PCR was carried out using the Agpath ID One-Step RT-PCR system (Ambion, USA). Multiplex sets of primers and Taq-Man probes are described in Table 1. The reaction mixture contained 5 μl of RNA template, 590 nM primers, 140 nM probe, 10 μl of 2x reaction buffer, 0.8 μl of enzyme Mixture, and RNase-free water with a final volume of 20 μl. One-step multiplex real-time RT-PCR was performed on an ABI 7500Fast (Applied Biosystems). The thermocycler conditions included a reverse transcription step at 50°C for 30 min, and an activation hot start DNA Taq polymerase at 95°C for 10 min, followed by amplification that was performed during 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 55°C for 30 sec. Multiple fluorescent signals were obtained once per cycle upon completion of the extension step. Data acquisition and analysis of the real-time PCR assay were performed using SDS software Version 1.4 (Applied Biosystems).

Specificity and sensitivity

Analytical specificity was evaluated by cross-reactivity with other subtypes of influenza viruses, respiratory viruses and bacteria (Table 2). The analytical sensitivity was tested using 10-fold serial diluted viral RNAs in triplicate, and the copy numbers of viral RNA were determined according to M (influenza A) and NP (influenza B) gene standards. In order to generate standard RNA transcripts for the sensitivity test, in vitro transcription was carried out using a MEGAscript T7 kit (Ambion) according to the manufacturer’s instructions. The concentration of the in vitro transcribed RNAs was calculated by measuring absorbance at 260 nm and the rests of RNAs were kept at -70°C until use.

WHO external quality assessment program (EQAP) panels

The assays were introduced to the WHO 10th EQAP panel which was comprised of 10 RNA samples and 2 inactivated viruses and that had been received from the WHO EQAP team in Hong Kong SAR.
Clinical specimen evaluation

The clinical throat swab specimens from outpatients with acute respiratory symptoms were collected from September 2008 to October 2010 through the Korea influenza surveillance system. A total of 319 specimens were tested retrospectively for evaluation of assay performance. Among them, 159 were influenza virus culture positive (seasonal 19 A/H1, 47 A/H3, 63 A/H1pdm09 and 30 B viruses) and 160 were culture negative. These samples were confirmed by real-time RT-PCR described here after viral culture. In addition, this assay was introduced to nationwide surveillance from September 2010 to August 2011, and 11,963 throat swab specimens were collected from sentinel clinics in 10 provinces and 7 metropolitan cities, and were evaluated at provincial health institutes. Informed consent was obtained from the patients or the parents of minor patients.

Results

Analytical sensitivity and specificity

The analytical sensitivities of each assay were determined by using triplicate 10-fold serial diluted viral RNAs of each virus ranging from 9.4 to 9.4×10^5 copies for A/H1, 4.8 to 4.8×10^5 copies for A/H3, 3.4 to 3.4×10^5 copies for influenza B, 1.9 to 1.9×10^5 copies for A/H5 and 2.5 to 2.5×10^6 copies for A/H1pdm09 virus. The limits of detection were as low as 1, 4.8, 1.9, 2.5 and 3.4 copies per reaction, corresponding to A/H1, A/H3, A/H5, A/H1pdm09 and B viral RNAs, respectively (Fig. 1). The specificity of each assay was evaluated by testing other respiratory viruses, including parainfluenza viruses 1/2/3, RSV, respiratory syncytial virus, coronavirus 229E/OC43, rhinovirus, enterovirus, human bocavirus and adenovirus. In addition, respiratory pathogenic bacteria such as *Haemophilus influenzae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* were assessed by applying approximately 200 ng of DNAs (Table 2). No cross-reaction was observed against those pathogens and among different influenza subtypes.

WHO EQAP panel evaluation

The assay performance was evaluated using the WHO 10th EQAP panel. Seven of the 10 RNA panels were identified as influenza A viruses: 4 A/H5, 2 A/H1 (2009) and 1 A/H3 and the remaining 3 panels were identified as being 2 influenza B viruses and 1 negative. Two inactivated virus panels were determined to be A/H3 (Table 3). All results were consistent with intended results.

Clinical specimen evaluation

The clinical performance of the assays was tested by using 319 throat swab samples, including 129 influenza A (19 H1, 47 H3 and 63 H1pdm09) and 30 influenza B samples pre-

Table 3. Evaluation of the multiplex real-time RT-PCR using the 10th WHO EQAP panel

| EQAP panel | Expected result$^a$ | A/M | A/H5 | A/H1(2009) | A/H3 | A/H1 | B | Copies / µl | Clade |
|------------|---------------------|-----|------|------------|------|------|---|-------------|-------|
| 2011-11    | B                   | -   | -    | -          | -    | -    | 26.9 | 5.67×10^4   | 2.3.2 |
| 2011-12    | A/H5                | 27.8| 27.6 | -          | -    | -    | -   | 1.149×10^2 | 2.3.4 |
| 2011-13    | B                   | -   | -    | -          | -    | -    | 25.7 | 1.990×10^4 | 2.3.4 |
| 2011-14    | A/H5                | 31.3| 31.2 | -          | -    | -    | 2.462×10^2 | 2.3.2 |
| 2011-15    | A/H3                | 31.6| -    | 31.6       | -    | -    | 7.180×10^4 | 2.3.4 |
| 2011-16    | A/H5                | 25.2| 24.8 | -          | -    | -    | 4.107×10^4 | 2.3.4 |
| 2011-17    | A/H1pdm09           | 30.7| -    | 29.2       | -    | -    | 4.948×10^4 | 2.3.2 |
| 2011-18    | A/H5                | 27.8| 27.0 | -          | -    | -    | 9.142×10^4 | 2.3.4 |
| 2011-19    | Neg                 | -   | -    | -          | -    | -    | -   | -           | -     |
| 2011-20    | A/H1pdm09           | 30.6| -    | 29.7       | -    | -    | 3.407×10^4 | 2.3.4 |
| V03-2011   | A/H3                | 36.3| 35.2 | -          | -    | -    | 1.310×10^4 | 2.3.4 |
| V04-2011   | A/H3                | 36.2| -    | 34.8       | -    | -    | 8.310×10^4 | 2.3.4 |

$^a$ WHO External Quality Assessment Program

$^b$ Each test was performed in triplicate and results were expressed by mean Ct value.

Table 4. The performance of multiplex Real-time RT-PCR with 319 clinical specimens

| Virus          | Viral culture$^a$ | Multiplex Real-time RT-PCR | Assay performance$^b$ |
|----------------|-------------------|-----------------------------|------------------------|
|                | Positive | Negative | Positive | Negative | PPV$^c$ | NPV$^d$ | Sensitivity | Specificity |
| Influenza A    | 129      | 190      | 119      | 200      | 100%   | 95.0%   | 92.2%       | 100%       |
| Influenza B    | 30       | 289      | 30       | 289      | 100%   | 100%    | 100%        | 100%       |
| A/H1           | 19       | 300      | 19       | 300      | 100%   | 100%    | 100%        | 100%       |
| A/H3           | 47       | 272      | 40       | 279      | 100%   | 97.4%   | 85.1%       | 100%       |
| A/H1pdm09     | 63       | 256      | 60       | 259      | 100%   | 98.8%   | 95.2%       | 100%       |
| Total (n = 319)| 159      | 160      | 149      | 170      | 100%   | 94.1%   | 93.7%       | 100%       |

$^a$ Virus culture was performed on MDCK cells.

$^b$ The A/H5 assay test was excluded.

$^c$ Positive predictive value

$^d$ Negative predictive value
viously cell culture positive, and 160 negative samples. Of these, the multiplex RT-PCR assays subtyped 149 samples which were consistent with viral culture as 119 influenza A (19 H1, 40 H3 and 60 H1pdm09) and 30 influenza B virus. However, the assays could not subtype 10 (7 A/H3 and 3 A/H1pdm09) viral culture positive samples. With viral culture as the reference, the overall positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity, except for the A/H5 assay, was 100%, 94.1%, 93.7%, and 100%, respectively (Table 4).

During the 2010–2011 season, a total of 11,963 throat swab samples collected through the Korea influenza surveillance system were tested, and 1,959 (16.3%) influenza A viruses (1,651 H1pdm09 and 308 H3) and 17 (0.1%) influenza B viruses were identified. However, no seasonal A/H1 virus which had circulated before the pandemic 2009 H1N1 virus was detected in these samples. The age distribution of the 1,976 influenza positive patients was 148 (7.4%) 0–2 years; 366 (18.5%) 3–6 years; 700 (35.4%) 7–19 years; 572 (28.9%) 20–49 years; 152 (7.6%) 50–64 years; 38 (1.9%) >64 years. The male to female ratio was 48.0% and positive rate of the vaccinated group was 21.1% (418 / 1,976) among influenza positive patients. The major clinical symptoms were fever and respiratory complications.

## Discussion

In Korea, the nationwide human influenza surveillance system adopted viral culture followed by conventional RT-PCR for human influenza virus detection and subtyping, until the 2009 pandemic outbreak. However, these methods are laborious, time-consuming and hard to standardize among the laboratories in local public health institutes which participate in surveillance. As the pandemic (H1N1)pdm09 virus emerged in April 2009, Korea NIC introduced rRT-PCR which was the WHO-recommended US CDC rRT-PCR Swine Flu Panel (WHO, 30 April 2009) to detect (H1N1)pdm09 virus. Although it showed invaluable performance during the early phase of the outbreak, it had some drawbacks (relatively low sensitivity to swH1 compared to the InfA assay, cross-reactivity with A/H5 of swInfluenzaA and single assay format) that made it undesirable to introduce for routine surveillance and diagnosis, in terms of cost-effectiveness and user convenience (Shu et al., 2011). Thereby, the development of an adequate and fine-tuned molecular detection system for influenza surveillance and diagnosis was required.

In the present study, the one-step multiplex rRT-PCR assays were developed and optimized for simultaneous detection of influenza type A and B and four subtypes of currently circulating human influenza, as well as avian influenza H5N1. Each assay was performed over a wide dynamic range and showed no cross-reactivity with other circulating respiratory viruses or bacteria. The assay also incorporates an internal control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe to screen possible PCR inhibitor or viral RNA extraction error. We also utilized the WHO EQAP panel for the evaluation of the A/H5 assay because there has so far been no H5N1 human infection case in Korea. The WHO panel consists of RNAs of seasonal influenza and avian influenza A/H5 with relatively low copy number, and inactivated seasonal influenza viruses (Table 3). For the Korea NIC, it is crucial to detect HPAI A/H5N1 human infection, because it has prevailed continuously in poultry farms since 2003 (Lee et al., 2005, 2008).

In comparison with other recently reported multiplex real-time RT-PCR assays, ours could detect currently circulating influenza viruses in humans, as well as avian A/H5 virus, implying that these are practical tools for influenza detection (Huber et al., 2011; Shisong et al., 2011). The detection limit of multiplex rRT-PCR described in this report is lower than 10 copies per reaction (Fig. 1), which suggests that these are comparable to other published assays (Chidlow et al., 2010; Chen et al., 2011). Moreover, we evaluated the assays by introducing them to nationwide influenza surveillance. Thereby, the assays could be validated in different work environments (17 public health institutes which participated in surveillance) with very large numbers of clinical specimens, and have shown excellent performance with reduced working time and costs compared to previous single type or two-step rRT-PCR assays (He, 2009; Nakauchi et al., 2011; WHO, 30 April 2009). However, the assays missed 10 influenza A (7 H3 and 3 H1pdm09) culture positive samples, because these showed signals which were near the detection limit (Ct values of >35 in A/M and subtypes) even after viral culture (Table 4).

One of our major concerns is laboratory contamination by PCR amplicons which cause false positive results, since conventional RT-PCR had previously been applied for influenza detection until the introduction of rRT-PCR to surveillance. Thus, viral RNA extraction, reaction mixture preparation and sample loading were carried out in a separated room to prevent possible contamination. We also tried to adjust assay sensitivity between the typing (multiplex set for influenza A or B type) and the subtyping assays to avoid inconclusive results in the influenza A sample that require further sequencing or tissue culture analysis.

In conclusion, well-balanced single-step multiplex rRT-PCR assays described here could be a useful tool for large scale surveillance and influenza diagnosis, as well as the detection of a possible HPAI A/H5 virus human infection.

## Acknowledgements

This work was supported by an intramural research funding 2010-N43001-00 from the Korea Centers for Disease Control and Prevention.

## Competing interests

None declared

## Ethic approval

This study was approved by Korea Center for Disease Control and Prevention institutional review board (IRB). The IRB approval number is 2011-06EXP-01-C.
References

CDC. 2009. Update: Infections with a swine-origin influenza A (H1N1) virus - United States and other countries. MMWR Morb. Mortal. Wkly. Rep. 58, 431–433.

Chen, Y., Cui, D., Zheng, S., Yang, S., Tong, J., Yang, D., Fan, J., Zhang, J., Lou, B., Li, X., and et al. 2011. Simultaneous detection of influenza A, influenza B, and respiratory syncytial viruses and subtyping of influenza A H3N2 virus and H1N1 (2009) virus by multiplex real-time PCR. J. Clin. Microbiol. 49, 1653–1656.

Chidlow, G., Harnett, G., Williams, S., Levy, A., Speers, D., and Smith, D.W. 2010. Duplex real-time reverse transcriptase PCR assays for rapid detection and identification of pandemic (H1N1) 2009 and seasonal influenza A/H1, A/H3, and B viruses. J. Clin. Microbiol. 48, 862–866.

Dawood, F.S., Jain, S., Finelli, L., Shaw, M.W., Lindstrom, S., Garten, R.J., Gubareva, L.V., Xu, X., Bridges, C.B., and Uyeki, T.M. 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N. Engl. J. Med. 360, 2605–2615.

Eccles, R. 2005. Understanding the symptoms of the common cold and influenza. Lancet Infect Dis. 5, 718–725.

He, J., Bose, M.E., Beck, E.T., Fan, J., Tiwari, S., Metallo, J., Jurgens, L.A., Kehl, S.C., Ledebor, N., Kumar, S., and et al. 2009. Rapid multiplex reverse transcription-PCR typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, including typing of novel swine origin influenza A (H1N1) virus, during the 2009 outbreak in Milwaukee, Wisconsin. J. Clínic Microbiol. 47, 2772–2778.

Huber, I., Campe, H., Sebah, D., Hartberger, C., Konrad, R., Bayer, M., Busch, U., and Sing, A. 2011. A multiplex one-step real-time RT-PCR assay for influenza surveillance. Euro Surveill. 16, pii= 19798.

Lee, Y.J., Choi, Y.K., Kim, Y.J., Song, M.S., Jeong, O.M., Lee, E.K., Jeon, W.J., Jeong, W., Joh, S.J., Choi, K.S., and et al. 2008. Highly pathogenic avian influenza virus (H5N1) in domestic poultry and relationship with migratory birds, South Korea. Emerg. Infect. Dis. 14, 487–490.

Lee, C.W., Suarez, D.L., Tumpey, T.M., Sung, H.W., Kwon, Y.K., Lee, Y.J., Choi, J.G., Joh, S.J., Kim, M.C., Lee, E.K., and et al. 2005. Characterization of highly pathogenic H5N1 avian influenza a viruses isolated from South Korea. J. Virol. 79, 3692–3702.

Nakauchi, M., Yasui, Y., Miyoshi, T., Minagawa, H., Tanaka, T., Tashiro, M., and Kageyama, T. 2011. One-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic influenza A/H1N1 2009, seasonal influenza A/H1N1, and seasonal influenza A/H3N2 viruses. J. Virol. Methods 171, 156–162.

Nakauchi, M., Yasui, Y., Miyoshi, T., Minagawa, H., Tanaka, T., Tashiro, M., and Kageyama, T. 2011. One-step real-time reverse transcription-PCR assays for detecting and subtyping pandemic influenza A/H1N1 2009, seasonal influenza A/H1N1, and seasonal influenza A/H3N2 viruses. J. Virol. Methods 171, 156–162.

Shisong, F., Jianxiong, L., Xiaowen, C., Cunyou, Z., Ting, W., Xing, L., Xin, W., Chunli, W., Renli, Z., Jinquan, C., and et al. 2011. Simultaneous detection of influenza virus type B and influenza A virus subtypes H1N1, H3N2, and H5N1 using multiplex real-time RT-PCR. Appl. Microbiol. Biotechnol. 90, 1463–1470.

Shu, B., Wu, K.H., Emery, S., Villanueva, J., Johnson, R., Guthrie, E., Berman, L., Warnes, C., Barnes, N., Klimov, A., and et al. 2011. Design and performance of the CDC real-time reverse transcriptase PCR swine flu panel for detection of 2009 A (H1N1) pandemic influenza virus. J. Clin. Microbiol. 49, 2614–2619.

Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J., and Fukuda, K. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. JAMA 289, 179–186.

WHO. 30 April 2009. Cdc protocol of real-time RT-PCR for influenza H1N1. Http://www.WHo.Int/csr/resource/publications/swineflu/cdcrealtimertpcr_swineh1assay-2009_20090430.Pdf.