Journal of Virological Methods 212 (2015) 76–79

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Quantitative RT-PCR evaluation of a rapid influenza antigen test for efficient diagnosis of influenza virus infection

Yuko Tsushima[a], Naoki Uno[a,b,*], Daisuke Sasaki[a], Yoshitomo Morinaga[a,b], Hiroo Hasegawa[a,b], Katsunori Yanagihara[a,b]

[a] Clinical Laboratory of Nagasaki University Hospital, Nagasaki 852-8501, Japan
[b] Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8501, Japan

Article history:
Received 17 April 2014
Received in revised form 20 October 2014
Accepted 28 October 2014
Available online 22 November 2014

Keywords:
Influenza virus
Rapid influenza antigen detection test
Quantitative RT-PCR

A B S T R A C T

Influenza virus infection is diagnosed in most cases using a rapid influenza antigen diagnostic test (RIDT). However, false-negative results are a major concern. By contrast, the nucleic acid amplification test offers high sensitivity and therefore can aid the interpretation of negative RIDT results. In this study, influenza viral loads were quantified with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using viral suspensions left over after RIDT, and the performance of both methods was evaluated. qRT-PCR detected as few as 10^3 copies/mL of influenza viruses A and B, whereas RIDT showed negative results for viral loads less than 10^7 and 10^5 copies/mL of influenza viruses A and B, respectively. These results indicate that small quantities of the virus that cause false-negative RIDT results can be detected efficiently with qRT-PCR follow-up. In addition, influenza A virus subtype was determined using qRT-PCR.

1. Introduction

The rapid influenza antigen diagnostic test (RIDT) using immunochromatography is used widely to detect viral antigens. RIDT requires no special skills or instruments and has a short turnaround time; therefore, it is a common point-of-care test for the detection of influenza virus infections (Welch and Ginocchio, 2010). Although its usual specificity exceeds 90%, its analytical sensitivity is variable, ranging from 10% to 80% (Chartrand et al., 2012; Uyeki et al., 2009). This variability may be attributable to differences in kit contents such as the medium or swab (Hurt et al., 2007; Luinstra et al., 2011; Smieja et al., 2010), patient age (Hurt et al., 2007; Ruest et al., 2003), the type of respiratory specimen (Agoritsas et al., 2006), and the time of sampling from illness onset (Ward et al., 2004). In particular, physical factors during sample collection have direct effects on the results (Smieja et al., 2010).

Conversely, the nucleic acid amplification test (NAAT) offers high sensitivity and it has therefore been developed for the detection of various viruses (Templeton et al., 2004). This technique also detects multiple targets in multiple samples (Wu et al., 2008; Yang et al., 2010). Despite these advantages, NAAT is not in general use in the clinical setting because it is complex and time-consuming. The Centers for Disease Control and Prevention (2013) recommend that further influenza testing be considered for patients who test negative with RIDT when community influenza activity is high and laboratory confirmation of influenza is desirable. However, further testing requires additional sample collection. From a pragmatic point of view, NAAT would be most beneficial if it were performed with the sample material left over after RIDT. Moreover, technical bias from the sample collection process would be eliminated if the same sample was used. In the present study, influenza viral loads were quantified using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in viral suspensions left over after RIDT, and the performance of both methods was evaluated.

2. Materials and methods

2.1. Clinical specimens

Two hundred fifty-five nasopharyngeal swab specimens were collected from patients at Nagasaki University Hospital between December 2012 and March 2013. All clinical specimens were examined using a Clearline Influenza A/B (H1N1) 2009 assay

Abbreviations: RIDT, rapid influenza antigen diagnostic test; NAAT, nucleic acid amplification test; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

* Corresponding author at: Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Tel.: +81 95 819 7574; fax: +81 95 819 7422.
E-mail address: nuno@nagasaki-u.ac.jp (N. Uno).

http://dx.doi.org/10.1016/j.jviromet.2014.10.019
0166-0934/

Table 1
Primer and probe sequences.

Primer and probe names	Oligonucleotide sequence (5′–3′)	Reference
Primers and probe for quantification of the influenza A matrix gene		
FLUAM-1F	AAGACCAATYYTGTCACCTCTGA	Centre for Health Protection (2009) and Ward et al. (2004), with modifications
FLUAM-1R	CAAAGCGTCTACGCTGCAGTCC	Centre for Health Protection, 2009 and Ward et al., 2004
UPL probe104	FAM-GTGCCCAG-TAMRA	Centre for Health Protection (2009) and Ward et al. (2004)
Primers and probe for quantification of the influenza B hemagglutinin gene		
INFB-1	AAATACGGTGGATTAAAYAAAAGCAA	van Elden et al. (2001), with modifications and Wu et al. (2008)
INFB-2	CCAGCAATAGCTCCGAAGAAA	van Elden et al. (2001) and Wu et al. (2008)
INFB probe	Cy5-CACCCATATTGGGCAATTTCCTATGGC-BHQ3	van Elden et al. (2001) and Wu et al. (2008)
Primers and probe for the influenza A hemagglutinin gene (H1 subtype)		
H1-247F	AACATGTTACCCAGGGCATTTCGC	Centre for Health Protection (2009)
H1-361R	GTGGTTGGGCCATGAGCTTTCTTT	Centre for Health Protection (2009)
H1-278P	Cy5-GAGGAACTGAGGGAGCAATTGAGTTCAG-BHQ3	Centre for Health Protection (2009), with modifications
Primers and probe for the influenza A hemagglutinin gene (H3 subtype)		
H3-293f F	ACCCTCAGTGTGATGGCTTCCAAA	Centre for Health Protection (2009)
H3-400R	TAAGGGAGGCATAATCCGGCACAT	Centre for Health Protection (2009)
H3-342P	HEX-ACGCAGCAAAGCCTACAGCAACTGTT-BHQ1	Centre for Health Protection (2009)

(Alere Medical, Tokyo, Japan) according to the manufacturer's instructions. Viral suspension that remained after RIDT was stored at −80 °C until RNA extraction.

2.2. Viral RNA preparation

Viral RNA was extracted directly from a 60-μL viral suspension using a QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) and digested with Amplification Grade DNase I (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Subsequently, the viral RNA was diluted with four volumes of RNase-free water and used as the template for qRT-PCR. For the positive control, AMPLIRUN Influenza A H1, H3, and B RNA controls were purchased from Vircell Microbiologists (Granada, Spain).

2.3. Primer and probe design for qRT-PCR

The primers used for influenza A virus quantification were modified partially from those described previously (Centre for Health Protection, 2009; Ward et al., 2004), and the probe was selected from the Universal Probe Library (Roche Applied Science, Mannheim, Germany). The primers and probes used for influenza A virus subtyping (Centre for Health Protection, 2009) and influenza B virus quantification were as described previously (van Elden et al., 2001; Wu et al., 2008), with modifications.

2.4. Preparation of viral RNA standards for viral load quantification

Complementary DNA was synthesized from AMPLIRUN Influenza A H3 and B RNA controls using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers (Invitrogen). PCR was performed with AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) to amplify a matrix gene of the influenza A virus and a hemagglutinin gene of the influenza B virus using primers FLUAM-1F/1R and INFB-1/2, respectively (Table 1). The PCR products were isolated and purified using a QIAquick PCR purification kit (QIAGEN) before cloning with a TA PCR Cloning kit (pTAC-1; Bio Dynamics Laboratory, Tokyo, Japan). Plasmids were recovered using a QIAprep Spin Miniprep kit (QIAGEN) and sequenced with a 3130 Genetic Analyzer (Applied Biosystems). The plasmid of influenza A was amplified further with PCR using primers FLUAM-1R and M13-T7_rev (5′-TTCTAATACGACTCACTATAGGGCGGATAACAATTTCACACAG-3′).
The PCR product was purified with the QIAquick PCR purification kit and used as the template for *in vitro* transcription. The plasmid of influenza B was digested by the restriction enzyme *Bam*HI (Takara Bio, Shiga, Japan) and purified using the QIAquick PCR purification kit. RNA was synthesized *via in vitro* transcription using a MEGAshortscript T7 kit (Ambion, Austin, TX, USA) and purified using a MEGAclear kit (Ambion). Purified RNA was separated *via* MultiNA capillary electrophoresis (Shimadzu, Kyoto, Japan), and a single band was confirmed.

2.5. qRT-PCR

One-step RT-PCR was performed using LightCycler 480 RNA Master Hydrolysis Probes (Roche Applied Science) in 20-μL reaction mixtures containing 5 μL diluted viral RNA, 7.3 μL LightCycler 480 RNA Master Hydrolysis Probes (2.7 × conc.), 1.3 μL activator, 1 μL enhancer (20 × conc.), 0.5 μM primer (each), and 0.25 μM probe (each). The primers and probes used are shown in Table 1. RT-PCR conditions were 63 °C for 3 min and 95 °C for 30 s, followed by 45 cycles of 95 °C for 10 s and 58 °C for 30 s. Standard curves were drawn from serial dilutions of viral RNA standards. For positive RIDT samples, either the influenza A or the influenza B virus was quantified. For negative RIDT samples, the influenza A and B viruses were quantified separately. qRT-PCR was performed using both H1 and H3 primers for positive RIDT samples to subtype the influenza A virus biplex. For negative RIDT samples, qRT-PCR was performed separately for H1 and H3 subtyping because the biplex reaction was found to be less sensitive than the monoplex reaction.

3. Results

3.1. Comparison of RIDT and qRT-PCR results

RIDT showed a positive result in 34 of 255 samples, which included 31 seasonal influenza A and 3 influenza B infections (Fig. 1). Pandemic influenza A 2009 (H1N1) was not detected in any sample. qRT-PCR was performed using these 34 positive RIDT samples and 77 randomly selected negative RIDT samples obtained from 101 patients. The median age of the patients was 42 years (range, 0–92 years). All patients, except two, had fever, respiratory symptoms, or both. The median time from illness onset to specimen collection was 1 day (range, 0 to >7 days). The two patients without fever and respiratory signs lived with persons infected with influenza virus. Viral RNA was readily amplified in all of the positive RIDT samples in concordance with the type of influenza virus. For the 77 negative RIDT samples, amplification was observed clearly in 22 samples for influenza A, three for influenza B, and one for both