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Rapid identification of oseltamivir-resistant influenza A(H1N1) viruses with H274Y mutation by RT-PCR/restriction fragment length polymorphism assay

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ABSTRACT

In the beginning of 2007–2008 Northern Hemisphere influenza season, the frequency of influenza A(H1N1) viruses bearing a previously defined oseltamivir resistance conferring amino acid change of Histidine to Tyrosine at position 274 (H274Y) of the neuraminidase (NA) increased dramatically. In order to rapidly detect such resistant viruses, an RT-PCR/restriction fragment length polymorphism (RT-PCR/RFLP) assay targeting amino acid 274 of the N1 NA molecule was developed to investigate the presence or absence of the H274Y mutation. The reverse primer was engineered to produce a BspHI site in the amplicon for oseltamivir-sensitive viruses with Histidine at position 274 (274H). A total of 50 influenza A(H1N1) specimens including 30 oseltamivir-sensitive and 20 oseltamivir-resistant ones submitted to the Centers for Disease Control and Prevention (CDC) during the 2007–2008 influenza season were successfully characterized by this assay. The assay was specific for grown A(H1N1) viruses and original clinical specimens, with a lower limit of detection of approximately 10 RNA transcript copies per reaction. Our RT-PCR/RFLP assay provides a simple, rapid and sensitive tool to monitor the emergence and spread of H274Y oseltamivir-resistant influenza A(H1N1) viruses.

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1. Introduction

Influenza viruses cause significant morbidity and mortality around the world. Each year, seasonal influenza infects 5–20% of the population, causing 250,000–500,000 deaths worldwide (Goodman et al., 2006). Although vaccination is the primary strategy for preventing influenza infections, antiviral agents against influenza viruses are effective for the prophylaxis and treatment of influenza. M2 ion-channel blockers (adamantane derivatives) and neuraminidase (NA) inhibitors are two main classes of antivirals which interfere with influenza virus infection. Both of these inhibitors are licensed in the United States and many other countries. Adamantane derivatives – amantadine and rimantadine – are not currently recommended in the U.S. due to the high proportion of resistance in circulating influenza A(H3N2) and A(H1N1) viruses (Bright et al., 2005, 2006).

Currently the U.S. Food and Drug Administration has approved two NA inhibitors, oseltamivir (Tamiflu) and zanamivir (Relenza), for treatment and prophylaxis of influenza. Oseltamivir is currently the most frequently prescribed drug for controlling influenza in the U.S. and some other countries (Fazio et al., 2008; Schunemann et al., 2007). This class of drugs acts by inhibiting the activity of neuraminidase, preventing the virus particle's release from infected cells (Moscona, 2005).

The H274Y mutation has been previously established as a genetic marker of resistance to oseltamivir among patients when infected with either influenza A(H1N1) or A(H5N1) viruses (Gubareva, 2004; Ward et al., 2005; Weinstock et al., 2003; WHO, 2006, 2008). In previous surveillance studies, the frequency of oseltamivir-resistant viruses with the H274Y mutation was low (0.33%) worldwide (Monto et al., 2006). At the beginning of 2007–2008 influenza season, however, an increased frequency of influenza A(H1N1) viruses with the H274Y oseltamivir resistance marker was detected in several countries in Europe and North America (ECDC, 2008; Lackenby et al., 2008; Nicoll et al., 2008; Sheu et al., 2008; WHO, 2008). The rise in oseltamivir-resistant influenza A(H1N1) viruses appears to be due to the spontaneous emergence and transmission of viruses with the H274Y mutation rather than selection as a result of increased oseltamivir use (ECDC, 2008). However, in the case of H5N1, resistance was only detected in the patients after drug treatment (De Jong et al., 2005). The emergence and extensive spread of oseltamivir-resistant influenza A(H1N1) viruses emphasizes the need for global forceful monitoring of drug resistance in influenza viruses. In recent years, several assays have been developed for the detection of NA inhibitors resistant mutants. Some of

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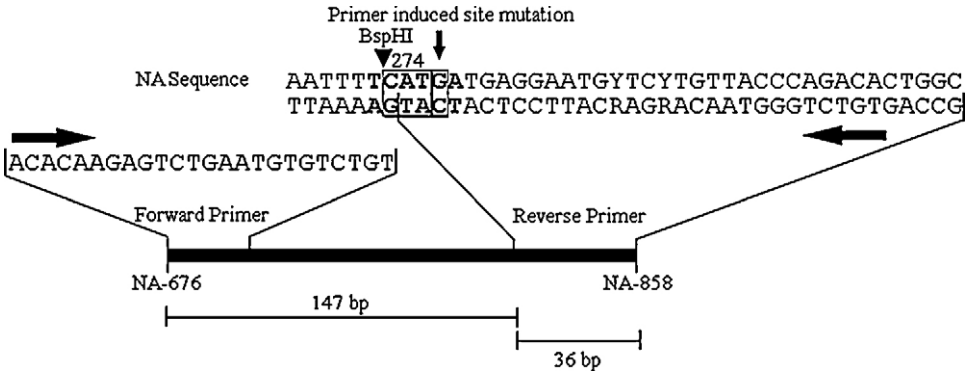

Fig. 1. Schematic drawing of the 183 bp RT-PCR amplification region and engineered BspHI restriction site in the NA gene of influenza A(H1N1) viruses. Primer locations and sequences are shown as black horizontal arrow. A to C change in the reverse primer, surrounded by a box, creates a BspHI restriction enzyme recognition sequence in viruses with 274H. The upside down triangle denotes the BspHI restriction site.

these assays are based on functional activity while others rely on sequence analysis of the NA gene.

In this study, we developed an RT-PCR/RFLP assay for the rapid differentiation of oseltamivir-sensitive and oseltamivir-resistant influenza A(H1N1) viruses based on the presence or absence of the H274Y change in the NA. We evaluated this assay with a collection of oseltamivir-resistant and -sensitive virus isolates and clinical specimens collected during the 2007–2008 U.S. influenza season.

2. Materials and methods

2.1. Viruses and RNA extraction

A total of 50 influenza A(H1N1) virus samples (39 virus isolates and 11 clinical specimens) submitted to the CDC during 2007–2008 influenza season by U.S. clinical and public health laboratories were used in this study. Other seasonal influenza viruses including influenza A(H3N2) and influenza B were used as controls for assay specificity. Viral RNA was extracted from 100 μl of each virus isolate or clinical specimen using MagNaPure LC extraction system (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol. All mutations in NA are given in N2 subtype numbering (Colman, 1989).

2.2. Assay design and RT-PCR amplification

To design the forward and reverse primers adjacent to the codon for amino acid 274, full length NA sequences of oseltamivir-resistant and -sensitive influenza A(H1N1) viruses obtained from NCBI Influenza Virus Resource and Los Alamos National Laboratory Influenza Sequence Database (LANL) from over 600 human influenza A(H1N1) viruses collected between 2007 and 2008 were aligned. The conserved region finder program ConFind was used to identify and design the primer for RT-PCR (Smagala et al., 2005).

The following primers were used for RT-PCR amplification (Fig. 1): forward primer F-676: 5′-ACACAAGAGTCTGAATG TGTCTGT-3′; reverse primer R-858: 5′-GCCAGTGTCTGGGTAACARGARCATTCC TCATCAT-3′. The introduction of underlined "C" in the R-858 primer to replace "A" at nucleotide 826 of the NA gene creates a BspHI restriction site next to the 274H codon. The RT-PCR was carried out using the SuperScript™III one-step kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The reaction mixtures contained 0.2 μM of each primer and 5 μl RNA (final volume 50 μl). The amplification was performed on a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hercules, CA) with the following thermocycling conditions: 30 min at 50 °C for reverse transcription, 2 min at 94 °C, followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 1 min at 68 °C, with a final extension of 68 °C for 10 min. A 183 bp product encompassing nucleotides 676–858 of the N1 NA gene was amplified.

2.3. RFLP analysis

For the RFLP analysis, 6 μl of each PCR amplicon was digested with 10 U BspHI (NEB, Ipswich, MA) by incubating at 37 °C for 1 h in a 20 μl reaction containing 1× reaction buffer 4 (NEB, Ipswich, MA). The amplicons and restriction fragments were analyzed by 2% agarose (GeneChoice, Frederick, MD) gel electrophoresis with GelRed™ staining (Biolium, Hayward, CA) and visualized by UV illumination.

2.4. NA inhibition assay and sequencing analysis

Confirmatory chemiluminescent NA inhibition assay and sequencing analysis were performed as previously described (Sheu et al., 2008).

2.5. Assay sensitivity

To evaluate the sensitivity of the RT-PCR assay, RNA transcripts were prepared from influenza A(H1N1) viruses using primers with the same sequence as the RT-PCR primers but with the addition of 5′-end T7 promoter sequence (underlined) in the forward primer to serve as template for RNA polymerase: F-676-T7: 5′-TAATACGACTCACTATAGGGACACAAGAGTCTGAATGTGTCTGT-3′. RNA transcripts were synthesized and purified using Megashortscript and MegaClear kits (Ambion, Inc., Austin, TX). The purified RNA transcripts were quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Following a 10-fold serial dilutions in 10 mM Tris–EDTA buffer, the transcripts were amplified using the primers described above for RT-PCR/RFLP and PCR products were analyzed by 2% agarose (GeneChoice, Frederick, MD) gel electrophoresis.

3. Results

A total of 39 MDCK cell cultured virus isolates were tested for the presence of the oseltamivir resistance conferring mutation H274Y. The gel electrophoresis of RT-PCR amplicons before and after digestion with BspHI are shown in Fig. 2. A 183 bp DNA fragment was amplified using the RT-PCR primer set. Strains without H274Y mutation were cleaved by BspHI, yielding two smaller segments 147 bp and 36 bp, respectively. The 36 bp fragment was too small to be clearly visualized on the gel. In contrast, oseltamivir-resistant strains were not cleaved by BspHI due to the mutation H274Y (CAT

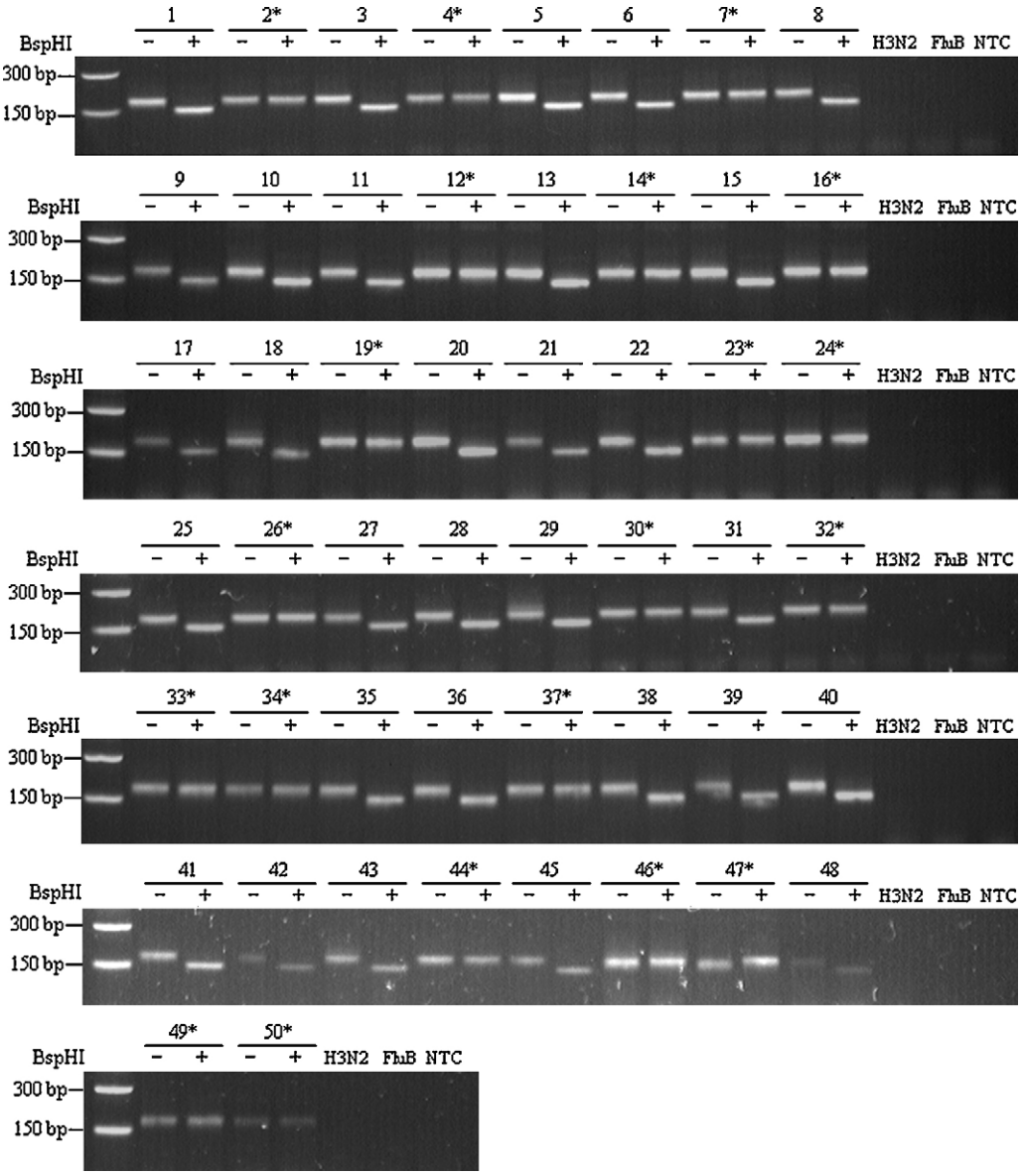

Fig. 2. Identification of influenza A(H1N1) viruses carrying the mutation at codon 274 in the NA gene by RT-PCR/RFLP analysis. Cultured virus isolates (numbers 1–39) and clinical specimens (numbers 40–50) were analyzed by the RT-PCR/RFLP assay. Influenza A(H3N2) and influenza B viruses were included as specificity controls. NTC: no template control. "−" denotes the RT-PCR amplicon in the absence of BspHI; "+" denotes the restriction fragments after BspHI digestion. Two fragments after BspHI digestion were 147 bp and 36 bp (36 bp band is not visible). Samples with H274Y change are marked by *.

to TAT). Of the 39 viruses tested, 24 were cleaved by BspHI and therefore classified as strains without H274Y mutation.

We also analyzed 11 clinical specimens (samples 40–50, Fig. 2). All were successfully amplified and analyzed by the RT-PCR/RFLP assay. Five of the 11 specimens were not digested by BspHI, indicating the presence of the oseltamivir resistance conferring mutation H274Y. As anticipated, RNAs from influenza A(H3N2) and influenza B were not amplified by the RT-PCR assay with the set of primers designed for the A(H1N1) viruses (Fig. 2).

The results of RT-PCR/RFLP assay were confirmed by NA inhibition assay for cell cultured virus isolates and by sequencing analysis of the NA gene for all tested samples with a 100% correlation (Table 1).

To further evaluate the sensitivity of the primers used in the RT-PCR assay, 10-fold serial dilutions of in vitro transcribed A(H1N1) RNA were tested. As shown in Fig. 3, as few as 10 copies of the in vitro transcribed RNA per reaction could be detected by RT-PCR.

Table 1
Summary of RT-PCR/RFLP results and sequence analysis for the H274Y mutation.

NAI phenotype[a]/sequencing analysis	RT-PCR/RFLP analysis (# of specimens)	
	Cleaved by BspHI	Not cleaved by BspHI
Sensitive/274H	30 (24 isolates + 6 originals)	0
Resistant/274Y	0	20 (15 isolates + 5 originals)

[a] NAI phenotype was determined using a chemiluminescent NA inhibition assay.

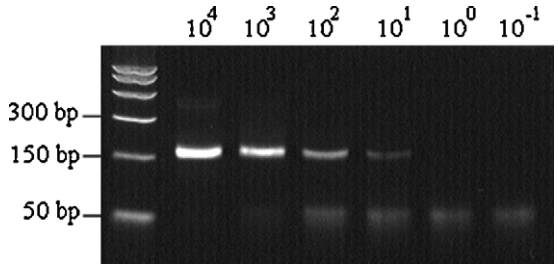

Fig. 3. 10-Fold serial dilutions of in vitro transcribed A(H1N1) RNA (see Section 2) ranging from 10^4 to 10^{-1} copy number per reaction were amplified by RT-PCR.

4. Discussion

The NA inhibition assay is currently the primary method used to screen for resistance to NA inhibitors (Gubareva et al., 2001, 2002; McKimm-Breschkin et al., 2003; Mungall et al., 2003, 2004; Monto et al., 2006; Wetherall et al., 2003; Zambon and Hayden, 2001). Following the NA inhibition assay, sequencing analysis is required to identify the genetic markers responsible for reduced susceptibility to NA inhibitors. Recently, pyrosequencing and real-time RT-PCR have become high throughput methods to screen influenza A viruses for known antiviral resistance markers (Bright et al., 2005; Deyde et al., 2007, 2008; Duwe and Schweiger, 2008; Chutinimitkula et al., 2007). These methods require well-trained personnel as well as specialized and expensive equipment, which may not be available in all laboratories. RT-PCR/RFLP analysis using inexpensive and commercially available endonucleases can be an alternative tool for those laboratories to monitor the emergence and spread of drug resistant viruses with established genetic markers, such as A(H1N1) H274Y oseltamivir-resistant viruses.

Our data have shown that the RT-PCR/RFLP assay developed in this study is a simple, specific, rapid, and accurate in detecting H274Y oseltamivir-resistant mutants among influenza A(H1N1) viruses. Resistant and sensitive viruses were correctly identified in all 50 influenza A(H1N1) virus samples examined, including 11 clinical specimens. Of note, the assay was sensitive enough to detect as few as 10 copies of transcribed RNA per reaction, demonstrating its utility for the screening of H274Y mutants from the RNA of clinical specimens thus reducing time needed to complete drug resistance analysis.

The RT-PCR/RFLP assay has certain limitations. It was developed specifically to detect the change in codon 274 in the NA of the human H1N1 subtype. Although, the H274Y mutation is the most common mutation thus far conferring oseltamivir resistance in the viruses of this subtype, other changes in the drug-targeting NA gene can also alter the drug susceptibility. Therefore, new RT-PCR/RFLP strategies will be necessary to detect other known or novel mutations conferring resistance to oseltamivir and/or zanamivir.

The surveillance of antiviral resistance is critical for making optimal drug treatment recommendations. The RT-PCR/RFLP assay described here presents a valuable and relatively inexpensive tool for the rapid identification of H274Y oseltamivir-resistant mutants in cultured viruses as well as in respiratory clinical material.

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Disclaimer: The findings and conclusions of this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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