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Pyrosequencing as a tool to detect molecular markers of resistance to neuraminidase inhibitors in seasonal influenza A viruses

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ABSTRACT

Pyrosequencing has been successfully used to monitor resistance in influenza A viruses to the first class of anti-influenza drugs, M2 blockers (adamantanes). In contrast to M2 blockers, resistance to neuraminidase (NA) inhibitors (NAIs) is subtype- and drug-specific. Here, we designed a pyrosequencing assay for detection of the most commonly reported mutations associated with resistance to NAIs, a newer class of anti-influenza drugs. These common mutations occur at residues: H274 (N1), E119 (N2), R292 (N2), and N294 (N2) in seasonal influenza A viruses. Additionally, we designed primers to detect substitutions at D151 in NAs of N1 and N2 subtypes. This assay allows detection of mutations associated with resistance not only in grown viruses but also in clinical specimens, thus reducing the time needed for testing and providing an advantage for disease outbreak investigation and management. The pyrosequencing approach also allows the detection of mixed populations of virus variants at positions of interest. Analysis of viruses in the original clinical specimens reduces the potential for introducing genetic variance in the virus population due to selection by cell culture. Our results showed that, in at least one instance, a D151E change seen in N1NA after virus propagation in cell culture was not detected in the original clinical specimen. Although the pyrosequencing assay allows high throughput screening for established genetic markers of antiviral resistance, it is not a replacement for the NA inhibition assays due to insufficient knowledge of the molecular mechanisms of the NAI-resistance.

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1. Introduction

Two classes of drugs, M2 blockers (adamantanes) and neuraminidase (NA) inhibitors (NAIs) are currently approved by the United States Food and Drug Administration for the prophylaxis and treatment of influenza A virus infections. Resistance acquired by viruses either in response to treatment or due to natural variation lessens the effectiveness of licensed antivirals. Monitoring of antiviral resistance is therefore an essential component of influenza virus surveillance. Adamantanes have been prescribed for prophylaxis and treatment of influenza infections for several decades. Genetic markers of resistance to this class of drugs in influenza A viruses are well established (Hay et al., 1986; Boivin et al., 2002). In 2005, a pyrosequencing assay for monitoring adamantane resistance was developed at the Centers for Disease Control and Prevention (CDC) and became the method of choice for high throughput screening for resistance to M2 blockers (Bright et al., 2005). This assay was instrumental in the timely detection of emergence and spread of adamantane-resistance among influenza A(H3N2) and A(H1N1) viruses in recent years (Barr et al., 2006, 2007; Bright et al., 2006b; Deyde et al., 2007; Saito et al., 2007a,b). Based on the results from this drug resistance surveillance, CDC issued a recommendation against use of adamantanes for treatment and prophylaxis of influenza infections in the U.S. until resistance subsides (Bright et al., 2006a).

Resistance to licensed NAIs, zanamivir and oseltamivir, is currently monitored using the NA inhibition assay (Gubareva et al., 2002; Mungall et al., 2003; Tisdale, 2000; Wetherall et al., 2003; Monto et al., 2006; Hurt et al., 2007). The use of the NA inhibition assay as a primary method for monitoring drug resistance has certain limitations (e.g. the need for virus propagation in cell culture prior to testing). It also requires NA subtyping and sequence analysis to identify genetic changes in the targeted enzyme. The analysis of resistance to this newer class of drugs is further complicated by the type/subtype specific nature of the molecular markers of resistance and uncertainty of their clinical relevance.

Until recently, the prevalence of resistance to NAIs has been low among field isolates (McKimm-Breschkin et al., 2003; Hayden et al., 2005; Monto et al., 2006; Mungall et al., 2003; Sheu et al.,

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2008; Zambon and Hayden, 2001). Several amino acid changes have been detected in the NA of influenza A and B viruses recovered from drug-treated patients (Abed et al., 2002; Gubareva et al., 2001; Hatakeyama et al., 2007; Ison et al., 2006; Kiso et al., 2004; Ward et al., 2005; Whitley et al., 2001; Escuret et al., 2008; Ferraris et al., 2005). The most commonly reported mutations associated with NAI-resistance in seasonal influenza A viruses are E119V, R292K, H274Y, and N294S (here and throughout the paper, we are using the universal N2NA numbering). The mutation H274Y was present in A(H1N1) viruses recovered from 7 of 43 oseltamivir-treated young children (Kiso et al., 2004). Mutation N294S was previously reported to be associated with resistance to zanamivir and/or oseltamivir in A(H3N2) viruses (Kiso et al., 2004; Yen et al., 2006). Influenza viruses carrying an amino acid substitution at residue 151 (D → N/G/E/V) often exhibit reduced susceptibility to NAIs in the chemiluminescent and/or fluorescent NA inhibition assay, although the role of changes at D151 in NAI-resistance remains uncertain and clinical relevance has not been demonstrated. The amino acid changes D151 to N, G, E, or V were previously reported among viruses that circulated from 1996 through1999 (McKimm-Breschkin et al., 2003). In more recent years, the D151V change was found in the NA of A/Montana/8/2007 (H3N2) which exhibited a 150-fold increase in IC_{50} value compared to its sensitive counterpart (Sheu et al., 2008). Additionally, the change D151A was detected in two viruses by conventional sequencing: A/Oman/6943/2005 (H3N2) and A/Canada/270/2007 (H3N2) with both viruses showing reduced susceptibility to zanamivir (Sheu et al., 2008).

The beginning of 2007–2008 influenza season was marked by an unprecedented circulation of A(H1N1) viruses resistant to oseltamivir in Europe (Lackenby et al., 2008) and elsewhere, including the U.S. (Sheu et al., 2008) and did not seem to be associated with use of the drug. The frequency of resistance ranged from 0% to 69% depending on the geographical location. The oseltamivir-resistance was conferred by the H274Y mutation (http://www.who.int/csr/disease/influenza/h1n1_table/en/). The increased frequency of H274Y mutants in circulation has caused concern because oseltamivir is the currently the most prescribed antiviral for control of influenza infections (Fazio et al., 2008; Schunemann et al., 2007). The emergence of oseltamivir-resistance among A(H1N1) viruses necessitates detailed epidemiological investigations and emphasizes the need for close monitoring of NAI-resistance in seasonal influenza viruses.

In response to the increased requirements for antiviral resistance surveillance, we developed a pyrosequencing approach for rapid detection of the most common markers of NAI-resistance in seasonal influenza A viruses, including the H274Y mutation in the NA of the N1 subtype.

2. Materials and methods

2.1. Viruses

The reference resistant viruses, A/Texas/36/91 (H1N1) with the H274Y mutation (Gubareva et al., 2001); and the oseltamivir-resistant E119V, A/Wuhan/359/1995 (H3N2)-like virus (Monto et al., 2006), with previously established markers of resistance were used for validation in the pyrosequencing assay. Virus isolates and clinical specimens from clinical and public health laboratories in the United States were submitted to the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the Influenza Division of CDC. Virus isolates of A(H1N1) subtype: A/Hawaii/21/2007, A/New Hampshire/02/2008, A/Pennsylvania/06/2008, A/Pennsylvania/18/2007, and A/Hawaii/28/2007 were used to analyze the sequence at codon 274. A/Pennsylvania/07/2008 (H274) and A/Illinois/08/2008 (H274Y) were used to assess the ability for the assay to detect mixtures of viral populations. A clinical specimen of the virus isolate A/Hawaii/28/2007 was also used to analyze sequences at two codons, 274 and 151.

Viruses of the A(H3N2) subtype: A/Bethesda/956/2006 and A/Illinois/01/2007 were used to analyze codons 292 and 294. The A/Illinois/01/2007 strain with E119 was used as the sensitive control. A(H3N2) viruses: A/Montana/08/2007, A/Oman/6943/2005, A/Hong Kong/4653/2005, and A/Canada/270/2007 were used for sequence analysis of codon 151. The NA sequences of viruses used in this study are available in the GenBank database. Monitoring for oseltamivir resistance was deemed to be surveillance, a public health practice, and exempt from CDC internal review board review.

Table 1
Human A(H1N1) and A(H3N2) NA pyrosequencing primers.

Primer	Sequence
A(H1N1): 151	
HuH1N1-151-F425	5′-ACAAACATTCAAATGGRACCG-3′
HuH1N1-151-R521-biot	5′-CTGACCATGCAACTGATTCAA-3′
HuH1N1-151-F425-seq	5′-ACAAACATTCAAATGGRACCG-3′
A(H1N1): 274	
HuH1N1-274-F770	5′-AGATCGAGAAGGGGAAGGTTACTA-3′
HuH1N1-274-R882-biot	5′-GTCYCTGCATACACACATCACT-3′
HuH1N1-274-F807-seq	5′-AAATGCACCCAAT-3′
A(H3N2): 119	
HuN2NA-F333-biot	5′-TGGGGACATCTGGGTGACA-3′
HuN2NA-R914	5′-ATATCTACTATGGGCCTATTGGA-3′
HuN2NA-119-R377-seq	5′-GGATCGCATGACACATA-3′
A(H3N2): 151	
HuN2NA-151-F423	5′-CAACGTGCATTCAAATGACAC-3′
HuN2NA-151-R567-biot	5′-CCAYGCTTTYCCATCRTG-3′
HuN2NA-151-F423-seq	5′-CAACGTGCATTCAAATGACAC-3′
A(H3N2): 292 and 294	
HuN2NA-F333-biot	5′-TGGGGACATCTGGGTGACA-3′
HuN2NA-R914	5′-ATATCTACTATGGGCCTATTGGA-3′
HuN2NA-292 and 294-R905-seq	5′-ATG GGC CTA TTG GAG CC-3′

2.2. Design of primers

Full length NA sequences obtained from the CDC sequence database and the Los Alamos Influenza sequence database (Macken et al., 2001) from 1200 human A(H1N1) viruses collected between 1968 and 2008 were aligned. A consensus N1NA sequence was generated using BioEdit Version 5.0.6 software (North Carolina State University). Similarly, a consensus sequence was generated based on alignment of 1300 NA sequences of the human A(H3N2) viruses. The consensus sequences were used to design all primer using Pyrosequencing Assay Design software (Biotage); however, in some cases, the primers were modified to accommodate degenerate nucleotides. The primers were synthesized at the CDC Biotechnology Core facility. Table 1 contains the list of the pyrosequencing primers designed in the present study.

2.3. RT-PCR and pyrosequencing

Viral RNAs were extracted either directly from clinical specimens or from viruses grown in MDCK cell cultures as previously described (Deyde et al., 2007). RT-PCR amplifications were performed using a SuperScript™III One-Step HI FI system (Invitrogen, Carlsbad, CA). Primers were used at 10 μM in a standard reaction mixture and amplification of 45 cycles. Biotinylated amplicons were purified in a series of buffers as described elsewhere (Bright et al., 2005). Single-stranded biotinylated DNAs were then transferred