Antiviral Research 81 (2009) 16–24

Contents lists available at ScienceDirect

Antiviral Research

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

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

Varough M. Deyde, Margaret Okomo-Adhiambo, Tiffany G. Sheu, Teresa R. Wallis, Alicia Fry, Nila Dharan, Alexander I. Klimov, Larisa V. Gubareva *

Influenza Division, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA

ARTICLE INFO

Article history:
Received 30 June 2008
Received in revised form 22 August 2008
Accepted 28 August 2008

Keywords:
Antiviral
Influenza A virus
Oseltamivir
Zanamivir
Pyrosequencing

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.

Published by Elsevier B.V.

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.,

* Corresponding author at: Mail Stop G-16, 1600 Clifton Road, Atlanta, GA 30333, USA. Tel.: +1 404 639 3204; fax: +1 404 639 0080.
E-mail address: lqg3@cdc.gov (L.V. Gubareva).

0166-3542/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.antiviral.2008.08.008

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

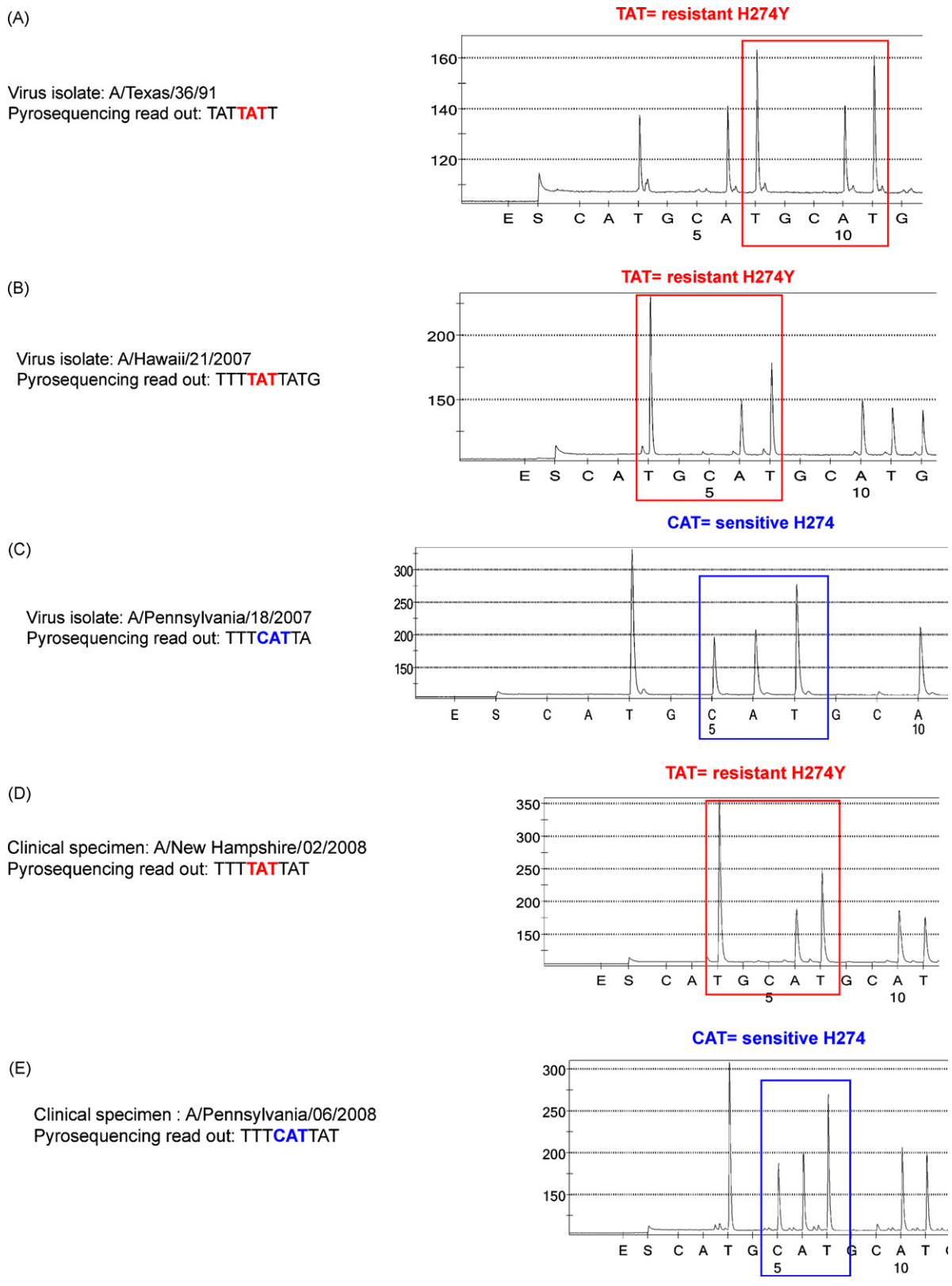
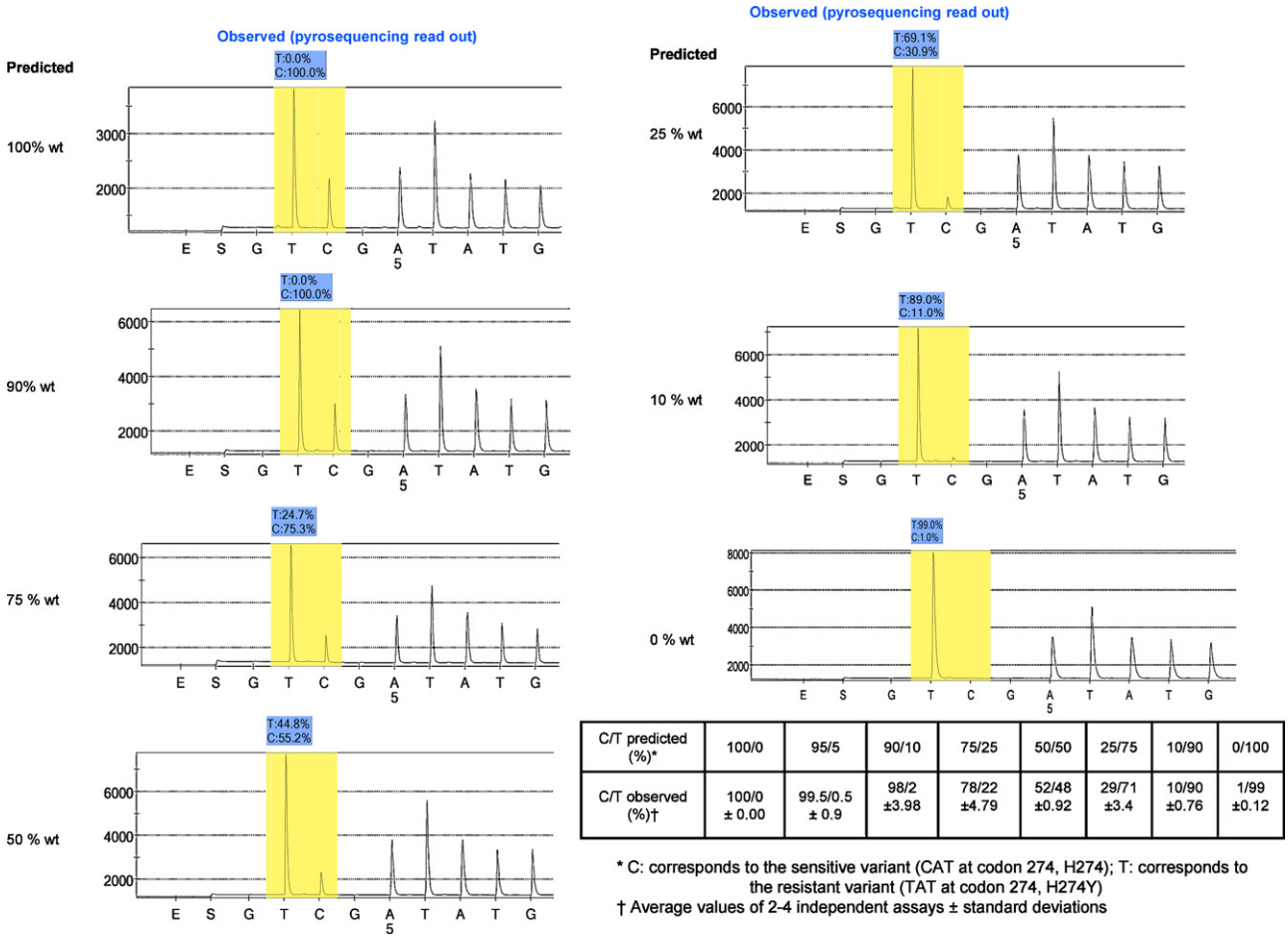


Fig. 1. Pyrosequencing allows detection of the most common mutations in NA in A(H1N1) viruses from clinical and viral isolates. Biotinylated PCR products were washed in a series of buffers and single-stranded DNA products were hybridized in a 96-well plate to mutation-specific sequencing primers used at 0.45 μM final concentrations in a 40 μl annealing buffer. Pyrosequencing was performed using pyrogold reagents according to Biotage recommendations. Panels A and B show the detection of the most common mutation known to confer resistance in A(H1N1) to oseltamivir, H274Y (CAT to TAT), in the reference virus A/Texas/36/91 as well as in a virus isolate collected during the 2007–2008 season, A/Hawaii/21/2007 (H1N1). The A/Pennsylvania/18/2007 (H1N1) virus that does not carry the mutation was included as a control (Fig. 1C). Panel D represents an example of H274Y mutation detection in a clinical specimen from which the A/New Hampshire/02/2008 (H1N1) virus was isolated in the 2007–2008 season; panel E represents an oseltamivir-sensitive virus, A/Pennsylvania/06/2008 virus, from a clinical specimen included as a sensitive control.



C/T predicted (%)*	100/0	95/5	90/10	75/25	50/50	25/75	10/90	0/100
C/T observed (%)†	100/0 ± 0.00	99.5/0.5 ± 0.9	98/2 ±3.98	78/22 ±4.79	52/48 ±0.92	29/71 ±3.4	10/90 ±0.76	1/99 ±0.12

* C: corresponds to the sensitive variant (CAT at codon 274, H274); T: corresponds to the resistant variant (TAT at codon 274, H274Y)
† Average values of 2-4 independent assays ± standard deviations

Fig. 2. Detection of mixed nucleotides (C or T) at codon 274 in N1NA using pyrosequencing. DNA amplicons from oseltamivir-sensitive A/Pennsylvania/07/2008 (H274: CAT) and oseltamivir-resistant A/Illinois/08/2008 (H274Y: TAT) were generated using the primers described in Table 1. The DNA concentrations of the PCR products were determined and mixed at indicated ratios. The samples were then pyrosequenced using the PSQ™96 HS platform. Examples of the pyrosequencing read out (highlighted in blue) are shown. Predicted values are on the left of panels. Averages of result from assay repeats and standard deviations are shown in the insert table.

to 40 μl annealing buffer containing pyrosequencing primers at 100 μM targeting the regions of interest. Pyrosequencing reactions were performed, and 45–60 nucleotide reads were obtained using the PSQ™96 MA platform, which generates nucleotide sequences for analysis. The sequences were then aligned and analyzed using DNAStar software as previously described (Bright et al., 2005). To indicate a position of mutation, the universal N2 numbering of the NA was used. To test the ability of the assay to detect mixtures of oseltamivir-sensitive and resistant virus variants in a single sample, the following experiment was conducted. Viral RNA from the two viruses, A/Pennsylvania/07/2008 (H274) and A/Illinois/08/2008 (H274Y), were independently extracted and used for amplification. The concentrations of the amplified DNAs were then determined and the DNA preparations were mixed at various proportions (100%/0% to 0%/100%). Samples were then pyrosequenced using either the PSQ™96 MA or the PSQ™96 HS platform to quantify the nucleotide mixtures at positions of interest (single nucleotide polymorphisms, SNPs); although the latter pyrosequencing platform does not generate sequence data for analysis.

The accuracy of sequences obtained from pyrograms was confirmed by comparison to the results of conventional sequencing.

2.4. Neuraminidase inhibition assay

The primary method used for screening susceptibility of seasonal influenza viruses to NAIs is chemiluminescent assay as described elsewhere (Sheu et al., 2008). In brief, the NA activity was determined before NAI assay by testing serial twofold dilutions of the virus. For the NAI, 25 μl of the dilutions of NA inhibitor were added to each well of a plate, to which 25 μl of virus dilutions were then added. Plates were pre-incubated with drug at 37 °C for 30 min. Ten microliters of the substrate diluted 1:1000 in NA-Star® assay buffer were added to each well and the plate was then incubated at room temperature for 30 min. Sixty microliters of NA-Star® accelerator were injected into each well of the 96-well plate and the luminescence read immediately for 0.5 s. Luminescence was measured using the multi-plate reader Victor3V (Perkin-Elmer, Shelton, CT) equipped with automatic injectors for accelerator.

3. Results

We used pyrosequencing to determine nucleotide sequences at four codons most commonly reported to contain markers of NAI-resistance in A(H1N1) and A(H3N2) viruses. To validate the designed primers and the overall pyrosequencing protocol, a set of reference viruses that either contained or lacked changes at one of those codons were tested.

Using the pyrosequencing approach, detection of the H274Y mutation (CAT to TAT) that confers oseltamivir-resistance in A(H1N1) viruses was demonstrated with the use of the oseltamivir-sensitive A/Texas/36/91 and its oseltamivir-resistant H274Y mutant (Gubareva et al., 2001). In addition, two virus isolates

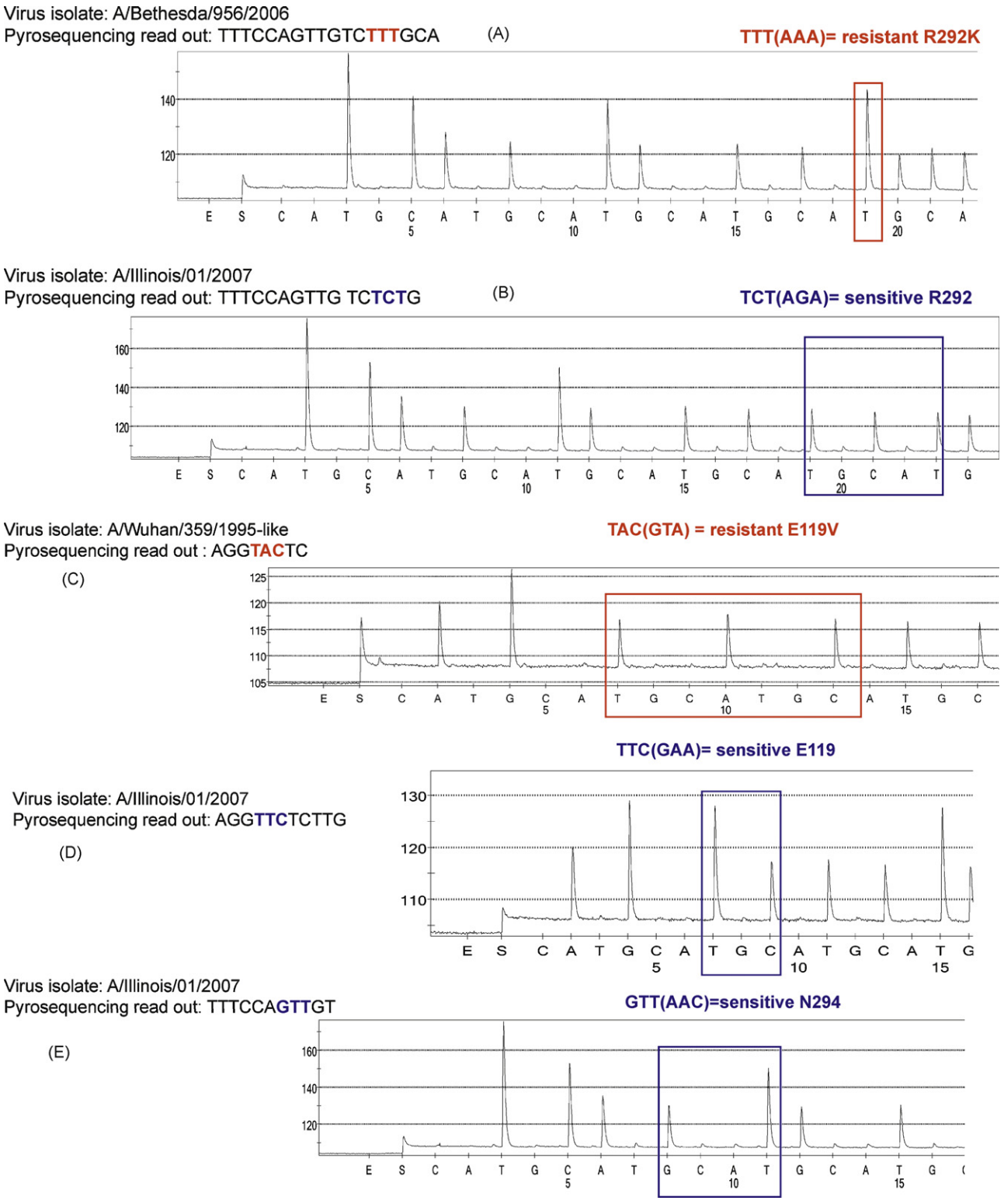


Fig. 3. Detection of R292K and E119V, the most common markers of resistance to NAIs in A(H3N2) viruses. Panels A and B show detection of mutation R292K. Pyrosequencing was performed as in Fig. 1. It should be noted that a reverse primer (HuN2NA-292 and 294-R905-seq), in Table 1 was used to sequence the region encompassing the 292 and 294 residues, therefore the sequences displayed are reverse complements of the original sequences. Viruses A/Bethesda/956/2006 and A/Illinois/01/2007 (both H3N2) were used to analyze mutations at residues 292. (A) shows that A/Bethesda/956/2006 has TTT, corresponding to AAA (K), at position 292, while the wild type A/Illinois/01/2007 (B) has TCT, equivalent to AGA (R), at position 292. The mutation R292K detected in A/Bethesda/956/2006 was previously shown to confer cross-resistance to both zanamivir and oseltamivir (Gubareva et al., 1997). Panels C and D show the detection of the mutation at codon E119. Similar to the R292K detection strategy, a reverse primer (HuN2NA-119-R377-seq), in Table 1, was used for sequencing the region containing the 119 codon; thus, the sequence displayed is a reverse complement of the original sequence. In the virus isolate A/Wuhan/359/1995 (H3N2), the sequence TAC that corresponds to GTA at codon 119 and codes for valine was detected (C). Drug resistance phenotypic assays of these virus clones showed that the mutation E119V resulted in a 115-fold reduction in sensitivity to oseltamivir compared to the wild type virus A/Illinois/01/2007 which has TTC corresponding to GAA (E119) (D). Although no virus with a N294S mutation was available for testing at this time, the designed primers for pyrosequencing allow testing of codon 294 in N2 (AAC) for A/Illinois/01/2007 (E).

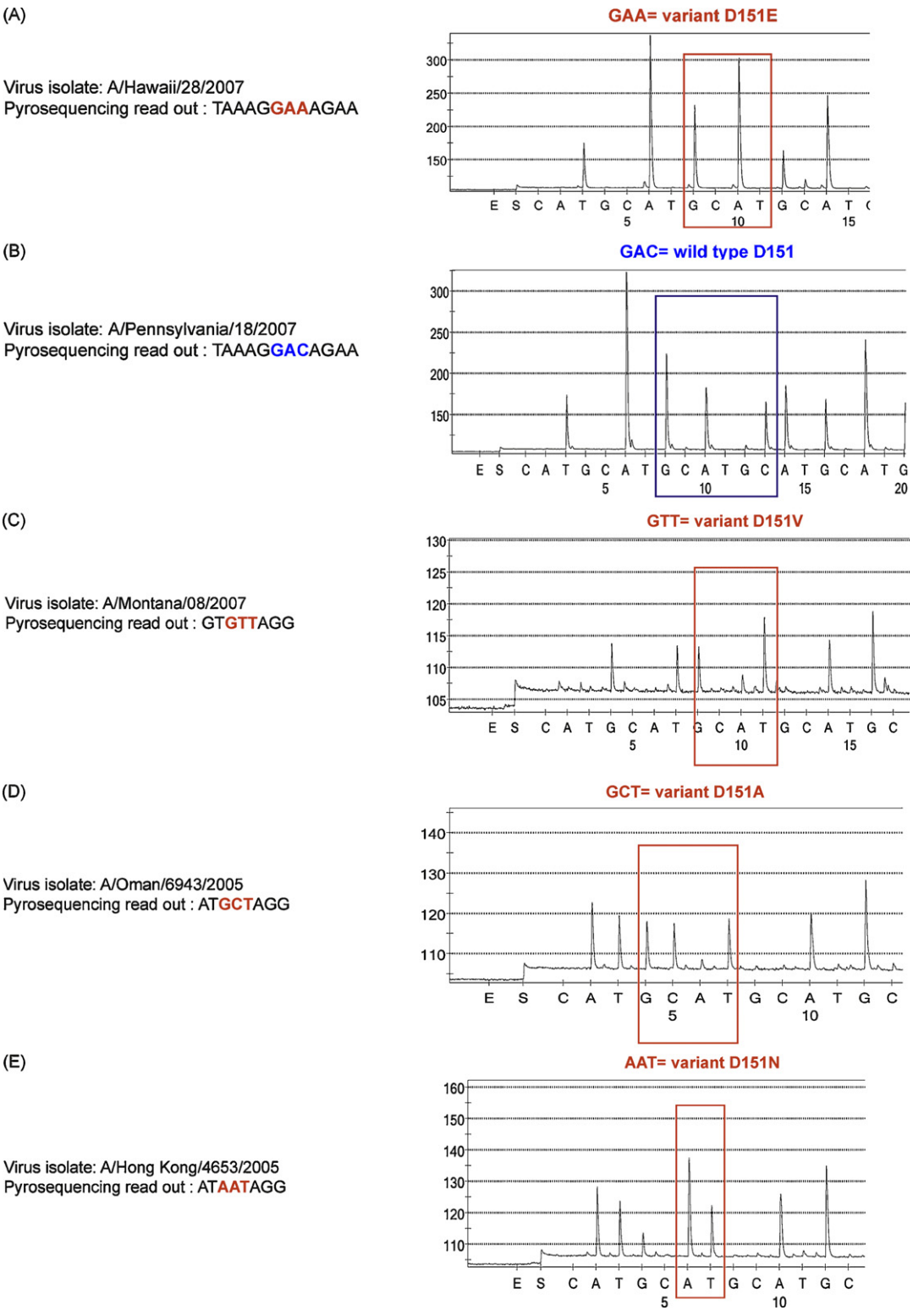


Fig. 4. Detection of substitutions at position D151 in both N1 and N2 neuraminidases. Changes at residue 151 have been previously reported to be associated with reduced sensitivity to zanamivir and/or oseltamivir. Here, we used pyrosequencing to detect changes at this position in several virus isolates that showed mild to extremely elevated IC_{50} values to zanamivir or oseltamivir in the NA inhibition assay. (A) shows detection of the mutation D151E (GAC to GAA) in an A(H1N1) virus, A/Hawaii/28/2007; the virus A/Pennsylvania/18/2007 did not contain this change and was used as a control (B). Among A(H3N2) viruses, we analyzed A/Montana/08/2007 virus and detected a GAT to GTT mutation at residue 151, corresponding to D151V (C), and also were able to detect GAT to GCT (D151A) change in A/Oman/6943/2005 virus (D). The mutation GAT(D) to AAT(N) at residue 151 in A/Hong Kong/4653/2005 (H3N2) was analyzed and detected (E). Interestingly, one virus, A/Canada/270/2007 (H3N2) had a mixture of wild type GAT (D) and mutant GCT (A) at residue 151 (F). In another case, the change GAC (D) to GAA (E) in an A(H1N1) virus at position 151 was not detected in the original clinical specimen prior to virus propagation in cell culture although the mutation was clearly present in the cultured virus isolate (G and H).

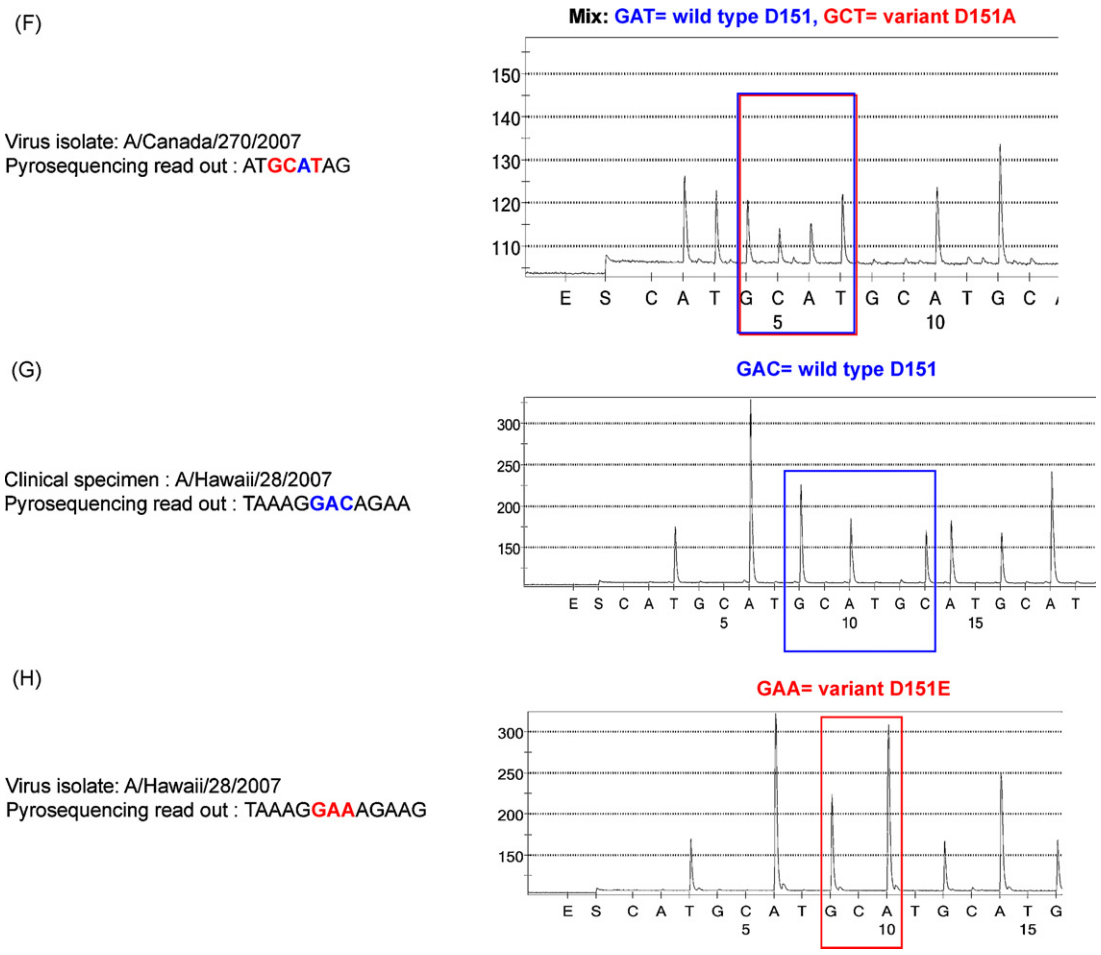


Fig. 4. (*Continued*).

collected during the 2007–2008 season (A/Hawaii/21/2007 and A/Pennsylvania/18/2007; Fig. 1A–C) were tested. Based on the results of the chemiluminescent NA inhibition assay; A/Texas/36/91 and A/Hawaii/21/2007 were resistant to oseltamivir, whereas, A/Pennsylvania/18/2007 was sensitive. Their IC_{50}s were 260.25, 109.5, and 0.25 nM, respectively. As anticipated, only the oseltamivir-resistant viruses contained the H274Y mutation.

To test the ability to detect the resistance-conferring mutation in the original clinical specimens, 171 samples collected from different locations during the 2007–2008 season were analyzed by pyrosequencing. Among those, 27 were found to contain the H274Y resistance marker while 134 did not (Fig. 1D and E). To optimize the RT-PCR conditions, the primer concentrations were increased up to 20 μM. This improved the amplification for some specimens; however, no RT-PCR products were obtained from 10 clinical specimens, most likely due to poor sample quality.

Influenza viral quasi species containing drug resistant and sensitive variants commonly emerge following treatment with NAIs (Gubareva et al., 1998; Boivin et al., 2002; Kiso et al., 2004; Le et al., 2005). Therefore, we assessed the assay ability to detect a mix of nucleotides at codon 274, associated with oseltamivir resistance. The PCR products of oseltamivir-sensitive, A/Pennsylvania/07/2008 (H274: CAT), and oseltamivir-resistant A/Illinois/08/2008 (H274Y: TAT) were quantified and five mixtures were prepared at ratio ranging from 10% to 90% each. The results showed that the observed values of these mixtures were close to the predicted ones (Fig. 2). For example, a predicted ratio of 75% C to 25% T was estimated at 78% C to 22% T.

As currently designed, the assay could not reliably detect the 5% of the resistant population in the mixture (Fig. 2, insert table).

Next, we tested the ability to detect the mutations in the N2 enzyme: R292K (AGA to AAA) (Fig. 3A and B) and E119V (GAA to GTA) (Fig. 3C and D). Although the A(H3N2) viruses carrying N294S mutation were not available for testing, we demonstrated that the designed primers allowed analysis of the sequence at codon 294 (AAC) (Fig. 3E).

Mutations at D151 have previously been detected in viruses exhibiting decreased susceptibility to zanamivir and/or oseltamivir in the NA inhibition assays (McKimm-Breschkin et al., 2003; Yen et al., 2006). In the present study, we demonstrated our ability to detect changes at D151 in both N1 (Fig. 4A and B), and N2 (Fig. 4C–F) enzymes. Mutations at residue D151 were observed in several viral isolates. Noteworthy, when original clinical specimens were tested prior to the virus isolation and propagation in cell culture, the changes at D151 were not detected in a number of instances. For example, D151E was seen in the A/Hawaii/28/2007 (H1N1) virus isolate by pyrosequencing (Fig. 4G) and conventional sequencing (data not shown) whereas, this change was not detected in the virus present in the original clinical specimen (Fig. 4E).

4. Discussion

In response to the alarming rise of oseltamivir-resistance in A(H1N1) viruses, we developed a pyrosequencing approach for detection of the most common markers of NAI-resistance among seasonal influenza A viruses. The pyrosequencing assay

strengthened U.S. surveillance for NAI-resistance by providing means for rapid screening of viruses for the presence of most common, well established markers of NAI-resistance. Noteworthy, the pyrosequencing assay permits detection of resistance markers in viruses in clinical specimens, prior to their isolation and propagation in cell culture, thus reducing time needed to complete the analysis. Moreover, the option to detect drug resistant mutants in the original clinical specimen is valuable because of the common presence of mixtures of virus quasi species (Abed et al., 2006; Gubareva et al., 1998, 2001). As demonstrated in the present study, pyrosequencing allows detection and quantitation of quasi species (sensitive and resistant) in the same samples.

Our data has shown the ability of the assay to detect the mixtures of sensitive and resistant viruses at ratios ranging from 10% to 90% (Fig. 2). Attempt in the present study to detect the presence of drug-resistant variant at 5% yielded unreliable outcome. Therefore, the assay in its current format is not suitable for detecting very small quasi species.

Pyrosequencing, as well as other sequence-based assays, cannot replace the enzyme activity inhibition assays at this time due to insufficient knowledge regarding markers of resistance to this newer class of drugs.

Emergence of mutants carrying H274Y in N1NA have been previously documented in patients treated with oseltamivir for seasonal A(H1N1) and avian A(H5N1) virus infections (de Jong et al., 2005; Hayden et al., 2005; Le et al., 2005). However, prior to the 2007–2008 influenza season, viruses with the H274Y mutation were rarely detected among community isolates. During season of 2007–2008, an increased proportion of A(H1N1) viruses with this mutation have been reported in several countries (http://www.who.int/csr/disease/influenza/h1n1_table/en/), and (http://www.cdc.gov/flu/weekly/), including the U.S. The pyrosequencing approach to detection of H274Y change, developed at CDC during the 2007–2008 season, has become a valuable tool in detection of such oseltamivir-resistant viruses among virus isolates and clinical specimens. The pyrosequencing assay can undoubtedly facilitate the global surveillance for this new resistant virus variant, however, it is essential to recognize that the pyrosequencing primers designed in the present study do not allow detection of the H274Y in oseltamivir-resistant A(H5N1) viruses due to substantial variability in the NA sequences of avian and human viruses (Russell et al., 2006).

While rarely reported among community isolates, changes at other residues have also been associated with zanamivir and oseltamivir-resistance in the NA inhibition assay: E119 (N2), R292 (N2), and N294 (N1 and N2). In addition, the assay allows detection of mutations at codon 151 in A(H1N1) and A(H3N2) viruses, however, different sets of primers are required due to the difference in the NA sequences of N1 and N2 subtypes. The residue D151 has been viewed as one of the key residues in the NA active site (Air et al., 1999; von Itzstein et al., 1996). Substitutions at this position were detected across types and subtypes, most often among A(H3N2) viruses, however not in all seasons (Monto et al., 2006). The relevance of substitutions at D151 for in vivo resistance has not been demonstrated, nevertheless, its presence in seasonal viruses needs to be monitored. In a recent mutagenesis study, the introduction of D151E in the N2NA background led to ~10-fold reduced susceptibility to oseltamivir (Yen et al., 2006). In the present study we detected the change D → E at position 151 only in the grown virus and not in the matching original specimen. This finding suggests a possible role for cell culture in selecting such NA variants, which might lead to altered susceptibility to NAIs. Pyrosequencing is well suited for exploration of such questions. Further studies addressing a potential role of cell cultures in selection of NA mutants, including those with substitution at D151, are underway.

Finally, the use of pyrosequencing assays allows the same viral RNA preparation to be used to detect resistance markers to both licensed classes of anti-influenza drugs, NAIs and M2 blockers. In addition, the pyrosequencing approach is relatively flexible and can be readily modified as soon as new markers of resistance are established or when substantial genetic variation occurs in the drug-targeted protein. Pyrosequencing is a technique best performed by trained professionals. However, the pyrosequencing platform can potentially be used for the diagnosis of pathogens or genetic disorders, and therefore is not limited to antiviral resistance detection in influenza. In conclusion, surveillance of resistance to antivirals, and especially NAIs, is critical because it provides information needed to make recommendation on optimal treatment and other public health policies for the control of influenza infections.

Conflict of interest

None declared.

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.

Funding source

This work was funded by the U.S. Centers for Disease Control and Prevention.

Acknowledgements

We thank Xiyan Xu, Henrietta Hall, Angela Foust, Amanda Balish, Wendy Sessions, Gregory Kocher, Jan Mabry, Rebecca Garten, Catherine Smith, John Barnes, Allison Myrick, and Michael Shaw from the Influenza Division, Centers for Disease Control and Prevention, for their contributions to the study, Jörn Winter, for fruitful discussions on statistical and sensitivity analyses; Deborah Litman, from Biotage for her help in the initial pyrosequencing primer design. We also thank U.S. State Laboratories, the National Influenza Centers participating in the Global Influenza Surveillance Network and the WHO Collaborating Centers for submitting their specimens for testing. Margaret Okomo-Adhiambo and Tiffany Sheu are grateful to the Oak Ridge Institute for Science and Education (ORISE) for providing fellowships.

References

Abed, Y., Baz, M., Boivin, G., 2006. Impact of neuraminidase mutations conferring influenza resistance to neuraminidase inhibitors in the N1 and N2 genetic backgrounds. Antiviral Ther. 11, 971–976.

Abed, Y., Bourgault, A.M., Fenton, R.J., Morley, P.J., Gower, D., Owens, I.J., Tisdale, M., Boivin, G., 2002. Characterization of 2 influenza A(H3N2) clinical isolates with reduced susceptibility to neuraminidase inhibitors due to mutations in the hemagglutinin gene. J. Infect. Dis. 186, 1074–1080.

Air, G.M., Ghate, A.A., Stray, S.J., 1999. Influenza neuraminidase as target for antivirals. Adv. Virus Res. 54, 375–402.

Barr, I.G., Hurt, A.C., Deed, N., Iannello, P., Tomasov, C., Komadina, N., 2007. The emergence of adamantane resistance in influenza A(H1) viruses in Australia and regionally in 2006. Antiviral Res. 75, 173–176.

Barr, I.G., Hurt, A.C., Iannello, P., Tomasov, C., Deed, N., Komadina, N., 2006. Increased adamantane resistance in influenza A(H3) viruses in Australia and neighbouring countries in 2005. Antiviral Res. 73, 112–117.

Boivin, G., Goyette, N., Bernatchez, H., 2002. Prolonged excretion of amantadine-resistant influenza a virus quasi species after cessation of antiviral therapy in an immunocompromised patient. Clin. Infect. Dis. 34, E23–E25.

Bright, R.A., Medina, M.J., Xu, X., Perez-Oronoz, G., Wallis, T.R., Davis, X.M., Povinelli, L., Cox, N.J., Klimov, A.I., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366, 1175–1181.

Bright, R.A., Shay, D., Breese, J., Klimov, A., Cox, N., Ortiz, J.R., 2006a. High levels of adamantane resistance among influenza A(H3N2) viruses and interim guidelines for use antiviral agents. MMWR Morb. Mortal Wkly. Rep. 55, 44–46 (United States, 2005–2006 influenza season).

Bright, R.A., Shay, D.K., Shu, B., Cox, N.J., Klimov, A.I., 2006b. Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. JAMA 295, 891–894.

de Jong, M.D., Tran, T.T., Truong, H.K., Vo, M.H., Smith, G.J., Nguyen, V.C., Bach, V.C., Phan, T.Q., Do, Q.H., Guan, Y., Peiris, J.S., Tran, T.H., Farrar, J., 2005. Oseltamivir resistance during treatment of influenza A (H5N1) infection. N. Engl. J. Med. 353, 2667–2672.

Deyde, V.M., Xu, X., Bright, R.A., Shaw, M., Smith, C.B., Zhang, Y., Shu, Y., Gubareva, L.V., Cox, N.J., Klimov, A.I., 2007. Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J. Infect. Dis. 196, 249–257.

Escuret, V., Frobert, E., Bouscambert-Duchamp, M., Sabatier, M., Grog, I., Valette, M., Lina, B., Morfin, F., Ferraris, O., 2008. Detection of human influenza A (H1N1) and B strains with reduced sensitivity to neuraminidase inhibitors. J. Clin. Virol. 41, 25–28.

Fazio, D., Laufer, A., Meek, J., Palumbo, J., Lynfield, R., Morin, C., Vivk, K., Baumbach, R., Mueller, J., Belflower, R., Long, C., Kamimoto, L., 2008. Influenza-testing and antiviral-agent prescribing practices—Connecticut, Minnesota, New Mexico, and New York, 2006–2007 Influenza Season. Morb. Mortal Wkly. Rep. 57 (3), 61–65.

Ferraris, O., Kessler, N., Lina, B., 2005. Sensitivity of influenza viruses to zanamivir and oseltamivir: a study performed on viruses circulating in France prior to the introduction of neuraminidase inhibitors in clinical practice. Antiviral Res. 68, 43–48.

Gubareva, L.V., Kaiser, L., Matrosovich, M.N., Soo-Hoo, Y., Hayden, F.G., 2001. Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. J. Infect. Dis. 183, 523–531.

Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G., 1998. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J. Infect. Dis. 178, 1257–1262.

Gubareva, L.V., Robinson, M.J., Bethell, R.C., Webster, R.G., 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. J. Virol. 71, 3385–3390.

Gubareva, L.V., Webster, R.G., Hayden, F.G., 2002. Detection of influenza virus resistance to neuraminidase inhibitors by an enzyme inhibition assay. Antiviral Res. 53, 47–61.

Hatakeyama, S., Sugaya, N., Ito, M., Yamazaki, M., Ichikawa, M., Kimura, K., Kiso, M., Shimizu, H., Kawakami, C., Koike, K., Mitamura, K., Kawaoka, Y., 2007. Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. JAMA 297, 1435–1442.

Hay, A.J., Zambon, M.C., Wolstenholme, A.J., Skehel, J.J., Smith, M.H., 1986. Molecular basis of resistance of influenza A viruses to amantadine. J. Antimicrob. Chemother. 18 (Suppl. B), 19–29.

Hayden, F., Klimov, A., Tashiro, M., Hay, A., Monto, A., Kimm-Breschkin, J., Macken, C., Hampson, A., Webster, R.G., Amyard, M., Zambon, M., 2005. Neuraminidase inhibitor susceptibility network position statement: antiviral resistance in influenza A/H5N1 viruses. Antiviral Ther. 10, 873–877.

Hurt, A.C., Selleck, P., Komadina, N., Shaw, R., Brown, L., Barr, I.G., 2007. Susceptibility of highly pathogenic A(H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. Antiviral Res. 73, 228–231.

Ison, M.G., Gubareva, L.V., Atmar, R.L., Treanor, J., Hayden, F.G., 2006. Recovery of drug-resistant influenza virus from immunocompromised patients: a case series. J. Infect. Dis. 193, 760–764.

Kiso, M., Mitamura, K., Sakai-Tagawa, Y., Shiraishi, K., Kawakami, C., Kimura, K., Hayden, F.G., Sugaya, N., Kawaoka, Y., 2004. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 364, 759–765.

Lackenby, A., Hungnes, O., Dudman, S.G., Meijer, A., Paget, W.J., Hay, A.J., Zambon, M.C., 2008. Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Eur. Surveill. 13 (5), pii: 8026.

Le, Q.M., Kiso, M., Someya, K., Sakai, Y.T., Nguyen, T.H., Nguyen, K.H., Pham, N.D., Ngyen, H.H., Yamada, S., Muramoto, Y., Horimoto, T., Takada, A., Goto, H., Suzuki, T., Suzuki, Y., Kawaoka, Y., 2005. Avian flu: isolation of drug-resistant H5N1 virus. Nature 437, 1108.

Macken, C., Lu, H., Goodman, J., Boykin, L., 2001. In: Osterhaus, A.D.M.E., Cox, N., Hampson, A.W.E. (Eds.), The Value of a Database in Surveillance and Vaccine Selection. Elsevier Science, Amsterdam, pp. 103–106.

McKimm-Breschkin, J., Trivedi, T., Hampson, A., Hay, A., Klimov, A., Tashiro, M., Hayden, F., Zambon, M., 2003. Neuraminidase sequence analysis and susceptibilities of influenza virus clinical isolates to zanamivir and oseltamivir. Antimicrob. Agents Chemother. 47, 2264–2272.

Monto, A.S., Kimm-Breschkin, J.L., Macken, C., Hampson, A.W., Hay, A., Klimov, A., Tashiro, M., Webster, R.G., Aymard, M., Hayden, F.G., Zambon, M., 2006. Detection of influenza viruses resistant to neuraminidase inhibitors in global surveillance during the first 3 years of their use. Antimicrob. Agents Chemother. 50, 2395–2402.

Mungall, B.A., Xu, X., Klimov, A., 2003. Assaying susceptibility of avian and other influenza A viruses to zanamivir: comparison of fluorescent and chemiluminescent neuraminidase assays. Avian Dis. 47, 1141–1144.

Russell, R.J., Haire, L.F., Stevens, D.J., Collins, P.J., Lin, Y.P., Blackburn, G.M., Hay, A.J., Gamblin, S.J., Skehel, J.J., 2006. The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 443, 45–49.

Saito, R., Li, D., Suzuki, H., 2007a. Amantadine-resistant influenza A (H3N2) virus in Japan, 2005–2006. N. Engl. J. Med. 356, 312–313.

Saito, R., Li, D., Suzuki, Y., Sato, I., Masaki, H., Nishimura, H., Kawashima, T., Shirahige, Y., Shimomura, C., Asoh, N., Degawa, S., Ishikawa, H., Sato, M., Shobugawa, Y., Suzuki, H., 2007b. High prevalence of amantadine-resistance influenza a (H3N2) in six prefectures in Japan, in the 2005–2006 season. J. Med. Virol. 79, 1569–1576.

Schunemann, H.J., Hill, S.R., Kakad, M., Bellamy, R., Uyeki, T.M., Hayden, F.G., Yazdanpanah, Y., Beigel, J., Chotpitayasunondh, T., Del Mar, C., Farrar, J., Tran, T.H., Ozbay, B., Sugaya, N., Fukuda, K., Shindo, N., Stockman, L., Vist, G.E., Croisier, A., Nagjdaliyev, A., Roth, C., Thomson, G., Zucker, H., Oxman, A.D., 2007. WHO Rapid Advice Guidelines for pharmacological management of sporadic human infection with avian influenza A (H5N1) virus. Lancet Infect. Dis. 7, 21–31.

Sheu, T.G., Deyde, V.M., Okomo-Adhiambo, M., Garten, R.J., Xu, X., Bright, R.A., Butler, E.N., Wallis, T.R., Klimov, A.I., Gubareva, L.V., 2008. Surveillance for Neuraminidase Inhibitor Resistance among Human Influenza A and B Viruses Circulating Worldwide from 2004-2008. Antimicrob. Agents Chemother. 52, 3284–3292.

Tisdale, M., 2000. Monitoring of viral susceptibility: new challenges with the development of influenza NA inhibitors. Rev. Med. Virol. 10, 45–55.

von Itzstein, M., Dyason, J.C., Oliver, Stuart, W., White, H.F., Wu, W.Y., Gaik, B., Kok, G.B., Pegg, M.S., 1996. A study of the active site of influenza virus Sialidase: an approach to the rational design of novel anti-influenza drugs. J. Med. Chem. 39, 388–391.

Ward, P., Small, I., Smith, J., Suter, P., Dutkowski, R., 2005. Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J. Antimicrob. Chemother. 55 (Suppl. 1), i5–i21.

Wetherall, N.T., Trivedi, T., Zeller, J., Hodges-Savola, C., Kimm-Breschkin, J.L., Zambon, M., Hayden, F.G., 2003. Evaluation of neuraminidase enzyme assays using different substrates to measure susceptibility of influenza virus clinical isolates to neuraminidase inhibitors: report of the neuraminidase inhibitor susceptibility network. J. Clin. Microbiol. 41, 742–750.

Whitley, R.J., Hayden, F.G., Reisinger, K.S., Young, N., Dutkowski, R., Ipe, D., Mills, R.G., Ward, P., 2001. Oral oseltamivir treatment of influenza in children. Pediatr. Infect. Dis. J. 20, 127–133.

Yen, H.L., Hoffmann, E., Taylor, G., Scholtissek, C., Monto, A.S., Webster, R.G., Govorkova, E.A., 2006. Importance of neuraminidase active-site residues to the neuraminidase inhibitor resistance of influenza viruses. J. Virol. 80, 8787–8795.

Zambon, M., Hayden, F.G., 2001. Position statement: global neuraminidase inhibitor susceptibility network. Antiviral Res. 49, 147–156.