

Influenza Antiviral Resistance Testing in New York and Wisconsin, 2006 to 2008: Methodology and Surveillance Data^{∇†}

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Received 15 October 2008/Returned for modification 12 December 2008/Accepted 17 March 2009

The need for effective influenza antiviral susceptibility surveillance methods has increased due to the emergence of near-universal adamantane resistance in influenza A/H3N2 viruses during the 2005-2006 season and the appearance of oseltamivir resistance in the influenza A/H1N1 virus subtype during the 2007-2008 season. The two classes of influenza antivirals, the neuraminidase inhibitors (NAIs) and the adamantanes, are well characterized, as are many mutations that can confer resistance to these drugs. Adamantane resistance is imparted mainly by a S31N mutation in the matrix gene, while NAI resistance can result from a number of mutations in the neuraminidase gene. During the 2007-2008 season, a neuraminidase mutation (H274Y) conferring resistance to the NAI oseltamivir emerged worldwide in the A/H1N1 virus subtype. Surveillance methodology and data from New York (NY) and Wisconsin (WI) for the 2006-2007 and 2007-2008 influenza seasons are presented. We used an existing pyrosequencing method (R. A. Bright et al., *Lancet* 366:1175–1181, 2005) and a modified version of this method for detection of adamantane resistance mutations. For NAI resistance mutation detection, we used a mutation-specific pyrosequencing technique and developed a neuraminidase gene dideoxy sequencing method. Adamantane resistance in the A/H3N2 virus samples was 100% for 2007-2008, similar to the 99.8% resistance nationwide as reported by the CDC. Adamantane resistance was found in only 1.2% of NY and WI A/H1N1 virus samples, compared to that found in 10.8% of samples tested nationwide as reported by the CDC. Influenza A/H1N1 virus H274Y mutants were found in 11.1% of NY samples for 2007-2008, a level comparable to the 10.9% nationwide level reported by the CDC; in contrast, mutants were found in 17.4% of WI samples. These results indicate the need for regional influenza antiviral surveillance.

Influenza virus is a highly contagious agent that can cause symptoms ranging from mild discomfort to severe respiratory disease and is a contributing factor in over 30,000 deaths annually in the United States alone (27). Currently, the major health-care strategies for controlling the spread of influenza entail two main approaches, vaccination and the use of antiviral drugs (21). Vaccines are the most effective method of limiting the spread of influenza and preventing symptoms (15). However, vaccine efficacy is limited to the strains selected for each year's vaccine. Since vaccine development and production typically require several months, prediction of the major seasonal strains can be difficult and is occasionally inaccurate. Also, vaccination compliance can vary from year to year, and outbreaks in long-term-care facilities can occur regardless of the residents' vaccination statuses. Thus, antiviral drugs may serve as the initial agents for the prevention and treatment of influenza. Currently, two classes of influenza antiviral drugs are approved for influenza prophylaxis and treatment in the United States: the M2 ion channel blockers (including aman-

tadine and rimantadine, collectively referred to as the adamantanes) and the neuraminidase inhibitors (NAIs) (oseltamivir and zanamivir).

Adamantanes are effective only against influenza type A virus; they function by blocking ion flow through the 97-amino-acid viral proton channel (the M2 protein of the matrix gene), which is necessary for replication (1). Adamantanes have been shown to be effective agents for the treatment of influenza symptoms as well as for prophylaxis (13, 23). However, recent publications have documented a rapid increase in the incidence of adamantane-resistant influenza virus strains during the 2005-2006 influenza season (4, 12). Adamantane resistance is associated with mutations in a short region of the M2 protein sequence encompassed in nucleotides 789 to 815 of the matrix gene. Thus far, five resistance mutations, in the codons for amino acids 26, 27, 30, 31, and 34, have been reported. The predominant circulating resistance mutation reported affects amino acid 31, resulting in a serine-to-asparagine (S31N) change (24, 26). During the 2005-2006 influenza season, the Centers for Disease Control and Prevention (CDC) found that more than 90% of A/H3N2 viruses circulating in the United States had the S31N adamantane resistance mutation and recommended that adamantanes not be used for treatment or prophylaxis of influenza until susceptibility can be reestablished (5). The results also indicated that only 4% of A/H1N1 viruses tested were adamantane resistant (12). In Asia during the same period, the levels of resistance were slightly higher:

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† Supplemental material for this article may be found at <http://jcm.asm.org/>.

[∇] Published ahead of print on 25 March 2009.

TABLE 1. Influenza virus matrix gene M2 primers

Method	Amplification primer		Pyrosequencing primer	
	Name	Sequence	Name	Sequence
Pyrosequencing	M2_F	5'-CAGATGCARCGATTCAAGTG ^a	M2seqF	5'-CAGATGCAGCGATTCAAGTG ^a
	M2_R Biotin	5'-biotin-AGTAGAAACAAGGTAGTTTTTACTC ^a		
	M2_F Biotin	5'- biotin -CAGATGCAGCGATTCAAGTG ^b	M2seqR	5'-AATCCACAATATCAAGTGCA
	M2_R	5'-AGTAGAAACAAGGTAGTTTTTACTC ^a		
Dideoxy	DDseqM2_F	5'-AGCACTACAGCTAAGGCTATGG ^c		
	M2_R	5'-AGTAGAAACAAGGTAGTTTTTACTC ^{a,c}		

^a From reference 4.

^b Modified from reference 4 by the addition of biotin, the substitution of G for R, and the insertion of an additional A (changes in boldface type).

^c Primer also used for dideoxy sequencing.

nearly all A/H3N2 and 15% of A/H1N1 viruses tested had an adamantane resistance mutation (12).

The use of NAIs has increased as a result of the decreased efficacy of adamantanes against such a large proportion of circulating influenza viruses. NAI antivirals are analogues of sialic acid, the biological neuraminidase (NA) substrate. The NAIs disrupt viral replication by occupying the NA active site, thus limiting the binding of sialic acid and the subsequent NA cleavage of host cell receptors (21, 28). Although currently not as common as adamantane resistance, NAI resistance can develop as a result of amino acid-altering mutations in the NA gene of influenza virus. Characterized NAI resistance mutations, distributed throughout the NA gene, result in catalytic or structural changes that affect the sialic acid binding site. Several studies have developed models predicting that the increased use of NAI antivirals, such as during an influenza pandemic, may result in an increase in circulating NAI-resistant viruses, which could greatly decrease the overall efficacy of antivirals used in a pandemic situation (10, 20). In Japan, oseltamivir is often prescribed for prophylactic therapy, as well as for treatment of influenza cases. The widespread use of the drug has been cited as a reason for higher rates of resistance in Japan, previously found to be as high as 18% in pediatric cases (18). In the early part of the 2007-2008 influenza season, an oseltamivir-resistant A/H1N1 virus strain was reported first in Norway and subsequently in other countries worldwide (19, 29). In many European nations where oseltamivir use is very limited, a high percentage of the 2007-2008 influenza season's A/H1N1 virus strains had the oseltamivir resistance mutation, H274Y. This finding may indicate that oseltamivir resistance can develop and spread, even in the absence of the selective pressure exerted by excessive use of the drug. Despite variable reports on the transmissibility of drug-resistant influenza viruses (3, 16), the H274Y mutant spread rapidly throughout Europe during the 2007-2008 season. Thus far, the oseltamivir-resistant virus has remained susceptible to zanamivir (7, 22).

Clearly, timely and reliable methods are needed to monitor the development of resistance to influenza antivirals if these drugs are to be used effectively in both seasonal and pandemic situations. Here, we describe such methods for monitoring the mutations that are known to increase resistance to either adamantanes or NAIs. Pyrosequencing allows the rapid screening of the critical region of the influenza A virus matrix gene, for

detection of specific adamantane resistance mutations, or of the NA gene, for detection of a specific NA resistance mutation (i.e., H274Y). For more-extensive NAI resistance genotypic analysis, a method that uses traditional Sanger sequencing and that is based on newly compiled databases of influenza virus sequences is described. We also report results, acquired with these methods, from surveillance programs conducted by two state public health laboratories during the 2006-2007 and 2007-2008 influenza seasons in New York (NY) and Wisconsin (WI).

MATERIALS AND METHODS

NY state. (i) Specimens and initial screening. In NY, influenza virus was obtained from patient respiratory samples and/or isolates from cultured patient samples. Total nucleic acid was extracted from primary samples using either a miniMag or an easyMag apparatus (bioMérieux, Durham, NC) according to the manufacturer's instructions. RNA was extracted from culture isolates with a Qiagen M48 (Qiagen, Germantown, MD) or a QIAamp viral RNA kit by using a QIAcube instrument. All primary samples were tested by real-time reverse transcription-PCR (RT-PCR) targeting the matrix gene of influenza A virus and the nonstructural gene of influenza B virus. Influenza A-positive specimens were also subtyped for hemagglutinin (HA) and NA by conventional RT-PCR.

(ii) RT-PCR. A Qiagen one-step RT-PCR kit was used throughout. Results were analyzed using Bio-Rad iQ5 software 2.0 (Bio-Rad, Hercules, CA). The crossing threshold (C_t) for each run was set by the user to the middle of the exponential portion of the amplification curve.

For conventional sequencing, RT-PCR was performed and samples were analyzed on 1% agarose gels. Bands of appropriate size were purified with Ultra-free-DA spin columns (Millipore, Bedford, MA) or with a Zymoclean gel DNA recovery kit (Zymo Research, Orange, CA) before sequencing.

(iii) Data set construction. Four different data sets were created for the optimization and selection of PCR primers, as well as for selection of additional sequences used to create positive control oligonucleotides. For targets within the M2 region of the influenza A virus matrix gene, full-length nucleotide gene sequences were selected from the Influenza Virus Resource of NCBI (2); this resource includes data from the Influenza Genome Sequencing Project of the National Institute of Allergy and Infectious Diseases and from GenBank. Where groups of identical sequences were present, representative sequences were selected. The final matrix gene data set included nucleotide sequences from 168 viruses collected worldwide over 72 years. Sequences were aligned, analyzed for conserved regions (>20 nucleotides homologous throughout the data set), and checked for errors using MEGA (Molecular Evolutionary Genetics Analysis) software version 3.1 (Center for Evolutionary Functional Genomics, Tempe, AZ) and Sequencher 4.5 (Gene Codes, Ann Arbor, MI) software. Pyrosequencing primer M2seqR and conventional sequencing primer DDseqM2_F (Table 1) were selected. This data set was also used to verify primer sequences that we selected for use from those described by Bright et al. (4) using the default settings

TABLE 2. Influenza virus NA gene RT-PCR and dideoxy sequencing primers

Type/subtype	Assay	Primer name	Primer sequence (5' to 3')
A/N1	RT-PCR/seq ^a	NAN1_F	AGCAGGAGTTTAAATGAATCCAA
A/N1	RT-PCR/seq	NAH1N1_R	CCACAAAAAGAAATGCTGCTC
A/N1	Sequencing	N1NAseq2_F	TCATGYTCTCACTTGGAAATGCAG
A/N1	Sequencing	N1NAseq3_R	CCATTAGGATCCCAAATCATCTC
A/N2	RT-PCR/seq	NAN2_F	ATGAATCCAAATCAAAAAGATAATAAC
A/N2	RT-PCR/seq	NAN2_R	ACAACAATACTGTTTGAGGTCCAC
A/N2	Sequencing	N2NAseq3_F	AAATGCRACACTGCTAGCTTCATTTA
A/N2	Sequencing	N2NAseq3_R	CCTGAGCACACATAACBGGAA
B	RT-PCR/seq	NAB_F	TATNCTCACATCAGGGGGAGT
B	RT-PCR/seq	NAB_R	TGTCCTGAGCCCATYAAACAGTAAAT
B	Sequencing	BNaseq2_F	ACCYTTTRTTGCTTGTGGACCAA
B	Sequencing	BNaseq3_R	CAAAGGGTCTTTTTGCTGTGTAA

^a RT-PCR/seq, used as both an RT-PCR and a sequencing primer.

of the CLUSTALW program within the BioEdit sequence alignment editor (Ibis Biosciences, Carlsbad, CA) and Sequencher 4.5.

Nucleotide sequences encoding the NA gene subtypes of A/N1, A/N2, and influenza B viruses were selected from the Influenza Virus Resource in GenBank, using the same criteria as above. Sequences from multiple countries, spanning approximately 88 years, were obtained in three separate groups: 65 NA sequences from A/H1N1 viruses, 126 NA sequences from A/H3N2 and A/H2N2 viruses, and 211 NA sequences from influenza B viruses. Sequences were aligned and checked as described above. Conserved regions were analyzed for selection of conventional sequencing primers for the NA gene of each group (Table 2).

All new primers chosen were analyzed using the Integrated DNA Technologies (Coralville, IA) SciTools website to calculate the percentage of GC content, check for possible homo- and heterodimerization, and determine melting temperatures. Primers were also analyzed for cross-reactivity by using the NCBI BLAST program.

(iv) **Pyrosequencing.** Pyrosequencing of samples from NY was done by following primarily the procedure of Bright et al. (4), with modifications to allow sequencing in both directions. For pyrosequencing of the forward strand, a second set of reactions, using a biotinylated forward primer (M2_F Biotin), a nonbiotinylated reverse primer (M2_R), and a new pyrosequencing primer, M2seqR (Table 1), were used to produce sequence complementary to that in the original Bright et al. reaction.

All NY M2 pyrosequencing was performed by the Wadsworth Center Molecular Genetics Core Facility, using a PyroMark ID pyrosequencer (Biotage AB, Uppsala, Sweden) according to the manufacturer's protocol in a cyclic dispensation mode (GATC) for 20 cycles. Sequences were analyzed with a 36-bp quality window. The software automatically designated each sample as "pass," "check," or "fail." "Pass" samples were unambiguous, with clearly defined peaks, while "fail" runs were clearly not usable, with no clear peaks above background or only nonsense sequences. "Check" runs required examination by the operator and were usually reliable sequences with some complicating factors, such as spurious peaks, added or deleted bases in homopolymer regions, and wide or double peaks, that could often be corrected by the operator.

Nucleic acid extracted from a cultured sample from a patient infected with H3N2 virus was used as an adamantane-sensitive control for pyrosequencing. Synthetic adamantane-resistant positive controls were designed by alteration of the A/Hong Kong/1/68 virus sequence so that it contained the following five amino acid codons known to confer adamantane resistance: L26F, V27A, A30T, S31N, and G34E (1, 25). Control sequences designated MUTA/HKcontF and MUTA/HKcontR (see Fig. S1 in the supplemental material) were designed to hybridize with pyrosequencing primers M2seqF and M2seqR, respectively, thus allowing sequencing in both directions. 5'-Biotinylated oligonucleotides of these control sequences were synthesized (Integrated DNA Technologies) with an additional 18 nucleotides of marker sequence. Control oligonucleotides (in binding buffer) were used at 0.625 μ M in the pyrosequencing protocol.

(v) **Dideoxy sequencing.** Dideoxy sequencing was performed by the Wadsworth Center Molecular Genetics Core Facility using an ABI BigDye Terminator version 3.1 cycle sequencing kit with an ABI 3130 or 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

For sequencing of the M2 region of the influenza A virus matrix gene, forward primer DDseqM2_F was selected from the matrix gene data set for use with primer M2_R (Table 1). RT-PCR with this primer pair produced a 456-bp amplicon encompassing nucleotides 572 to 1027 of the gene; this amplicon was

subsequently sequenced in both directions by using the same primers as for the amplification reaction.

For dideoxy NA gene sequencing, RT-PCR primer pairs for A/H1N1, A/H3N2, and influenza B viruses were chosen (from their respective data sets, as detailed above) to amplify approximately 1.3 kb of the NA gene (Table 2). The amplified segments encompassed approximately 92% of the gene and contained all reported sites of mutation previously shown to decrease susceptibility to NAIs, as well as regions considered to be potential sites of additional, as-yet-uncharacterized resistance mutations. Internal primers are shown in Table 2, along with the amplification primers. Sequences obtained from these reactions were then aligned with Sequencher software, and the complete consensus gene sequence was determined.

WI. (i) Specimens and initial screening. Influenza virus was obtained from patient respiratory samples and/or isolates from cultured patient samples. Total nucleic acid was extracted using a MagNA Pure LC apparatus (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. All primary samples were typed and HA subtyped using real-time RT-PCR. The NA subtype was inferred from the HA subtype through confirmation of a subset of samples, via sequencing at the CDC, to determine currently circulating influenza A subtypes. The NA subtype was further confirmed by successful human A/H1N1 virus-specific pyrosequencing of the NA gene.

(ii) **RT-PCR.** A Qiagen one-step RT-PCR kit (Qiagen, Inc., Germantown, MD) was used throughout.

(iii) **Pyrosequencing.** Pyrosequencing of the WI samples was done by using the same methods as for the NY samples (described above) for unidirectional pyrosequencing of both the M2 and NA genes of the A/H1N1 virus. For each reaction, the biotinylated RT-PCR product size was confirmed by using precast 2% agarose E gels (Invitrogen, Carlsbad, CA), by following the manufacturer's protocol. The remaining sample was used for pyrosequencing reactions. Adamantane- and NAI-sensitive and -resistant controls were selected and validated from nucleic acid extracted from cultured surveillance clinical specimens referred from participating clinical virology laboratories. Primer sequences for pyrosequencing detection of the H274Y mutation in the NA gene were obtained from Deyde et al. (11).

RESULTS

Validation of pyrosequencing methods. Both NY and WI initially validated the pyrosequencing methods for detection of adamantane resistance mutations, using cultured isolates and primary specimens from previous influenza seasons. Studies were also conducted in WI to determine the effect of previous freezing of specimens on test results. Of the 30 frozen influenza A/H3 samples tested from WI, 23 possessed the adamantane resistance mutation (data not shown): 1 from 2003-2004, 3 from 2004-2005, and 19 from 2005-2006. Acceptable sequences generated from each sample were in agreement for the tissue culture isolates and the primary specimens. All 30 frozen tissue-culture isolates resulted in a "pass" quality rating,

TABLE 3. NY influenza A virus adamantane resistance mutations, 2001 to 2007

Sample type	Influenza A virus subtype ^a	
	H1N1	H3N2
Primary specimens	0/16	18/22 ^c
Culture specimens	1/15 ^b	12/15 ^c
Total ^d	1/24	30/35

^a Data are the number positive/total number; all mutations detected were S31N.

^b Resistant H1N1 virus from a 2006 patient with a history of travel in Asia (see Results).

^c All resistant H3N2 virus samples were from 2005.

^d Viruses with both primary and culture results are counted only once in the total.

while 24 (80%) of the frozen primary specimens resulted in an acceptable “pass” or “check” quality rating. However, all six primary specimens that failed were older specimens, from 2003-2004 or 2004-2005, suggesting that RNA quality for pyrosequencing can be significantly compromised in primary specimens that have been frozen for an extended period of time.

Further studies were conducted to determine the real-time PCR C_t value, as an indicator of the viral RNA quantity sufficient to yield quality pyrosequencing results directly from primary specimens. In preliminary studies in WI, a C_t value of 26.0 was chosen as the cutoff for exclusion from pyrosequencing testing for the S31N adamantane resistance mutation (see Fig. S2A in the supplemental material). Four of 33 test samples had C_t values greater than 26 and would not have been pyrosequenced had the cutoff been used. Of the four, three had “fail” pyrosequencing results and one had a “check” result. A subsequent analysis of all 2006-2007 samples revealed that the excluded specimens (those with C_t values greater than 26) accounted for approximately 15% of all surveillance samples collected. Similar pyrosequencing validation studies in WI were conducted for detection of the H274Y NAI resistance mutation (see Fig. S2B in the supplemental material), but a cutoff value was not used, and all samples were pyrosequenced. Of 59 samples, 5 were rated “fail,” 8 were rated “check,” and 46 were rated “pass.” Only the pyrosequencing results that were rated “pass” gave usable results.

Analysis of the bidirectional adamantane resistance mutation pyrosequencing quality ratings for the 2006-2007 NY samples revealed that nearly all primary samples tested generated usable sequence, when tested directly. For NY samples, a cutoff C_t value was not used; all 91 primary specimens were tested. In the forward direction (see Fig. S2C in the supplemental material), all “pass” and “check” samples (82 and 8, respectively) gave usable sequences. Only two primary samples were not pyrosequenced successfully; however, for one of these “fail” samples, sequence was obtained using the reverse primer. Thus, only one primary specimen from the entire season did not give satisfactory results in at least one direction.

The NY validation also included a comparison of pyrosequencing and dideoxy sequencing results from 24 A/H1N1 and 35 A/H3N2 virus samples obtained during 2001 to 2007. The 24 A/H1N1 virus samples consisted of nucleic acid extracts from 16 primary specimens (7 with matched culture isolates) and 8

TABLE 4. Adamantane S31N resistance mutations detected in NY and WI specimens

Season/state	Influenza A virus subtype (%) ^a		
	H1N1	H3N2	Undetermined
2006-2007/NY	0/37 (0)	38/42 (90.5)	6/11 (54.5)
2006-2007/WI	0/107 (0)	29/39 (74.4)	NA
2006-2007/total, NY and WI	0/144 (0)	67/81 (82.7)	6/11 (54.5)
2007-2008/NY	0/44 (0)	101/101 (100)	NA
2007-2008/WI	1 ^b /42 (2.4)	44/44 (100)	NA
2007-2008/total, NY and WI	1 ^b /86 (1.2)	145/145 (100)	NA

^a Data are the number positive/total number (%). NA, not applicable.

^b The one adamantane-resistant A/H1N1 virus specimen (from a 2007 patient with a history of travel in Asia) was susceptible to oseltamivir.

culture isolates only, all from different patients. No known adamantane resistance mutations were found in H1N1 virus samples obtained from primary extracts (Table 3). One H1N1 virus from a culture extract obtained in September 2006 (no primary specimen extract was available) had a S31N mutation; however, the patient had recently traveled to China. The 35 A/H3N2 virus samples consisted of 22 primary specimens (two with matched culture isolates) and 13 culture isolates only, all from different patients. In 30 (86%) H3N2 virus samples, a S31N resistance mutation was detected (Table 3); of these, 18 were primary specimens and 12 were culture extracts for which no primary sample was available. No other known resistance mutations were detected in any of the samples. Dideoxy sequencing results were identical to pyrosequencing results in all cases (results not shown).

NA dideoxy sequencing. Using the primers in Table 2, we obtained high-quality sequence data for the NA genes of A/N1, A/N2, and B viruses directly from most primary samples, eliminating the need for time-consuming viral cultures in many cases. For NA N1 sequencing, 45 of 51 primary samples (88.2%) yielded usable data. For N2 sequencing, 84 of 91 primary samples (92.3%) gave satisfactory results, compared to the appropriate NA database. The influenza B virus NA primers resulted in successful sequencing of 49 of 73 samples (67.1%). The lower success rate with influenza B virus NA may have been related to sample freezing and thawing, given that most primary sample extracts had been frozen and thawed at least twice (for other testing) before the sequencing reactions. All cultured viral isolates gave acceptable sequencing results with the appropriate primer set.

Adamantane resistance surveillance. A total of 236 surveillance samples (primary specimens or culture isolates) identified as influenza A virus were successfully evaluated during the 2006-2007 influenza season for the presence of amino acid changes conferring resistance to the adamantanes, at the NY (90 samples) and WI (146 samples) reference laboratories (Table 4). Of these, 144 (61.0%) were subtyped as influenza A/H1 virus, 81 (34.3%) as influenza A/H3 virus, and 11 (4.7%) could not be subtyped. None of the A/H1 virus samples possessed

TABLE 5. Neuraminidase H274Y resistance mutations detected in NY and WI specimens

Season/state	Influenza type/subtype (%) ^a		
	A/H1N1	A/H3N2	B
2006-2007/NY ^b	0/18	0/17	0/19
2007-2008/NY ^b	5/45 (11.1)	0/92	0/45
2007-2008/WI ^c	8/46 (17.4)	ND	ND
2007-2008/total, NY and WI	13/91 (14.3)	0/92	0/45

^a Data are the number positive/total number (%). ND, not determined.

^b Genotyped by dideoxy sequencing of the NA gene.

^c Genotyped by H274Y-specific pyrosequencing.

resistance mutations, while 82.7% of the A/H3 virus samples and 54.5% of the nontyped samples had the S31N mutation.

During the 2007-2008 influenza season, the two reference laboratories successfully evaluated a total of 231 influenza A virus surveillance samples for the same adamantane resistance mutations (NY, 145 samples; WI, 86 samples). Of these, 86 (37.2%) were subtyped as influenza A/H1 virus, and 145 (62.8%) as influenza A/H3 virus. Adamantane resistance was observed for all influenza A/H3 virus samples, and only a single influenza A/H1 virus sample, associated with a patient's recent travel history to Southeast Asia, possessed the S31N mutation.

NAI resistance surveillance. A total of 54 influenza virus surveillance samples were successfully evaluated in NY during the 2006-2007 influenza season for the presence of mutations conferring resistance to the NAIs (Table 5). Of these, 18 (33.3%) were subtyped as influenza A/H1N1 virus, 17 (31.5%) as influenza A/H3N2 virus, and 19 (35.2%) as influenza B virus. None of these influenza virus samples possessed a NAI resistance mutation.

During the 2007-2008 influenza season, the two reference laboratories successfully evaluated 228 influenza virus surveillance samples (NY, 182 specimens; WI, 46 specimens) for the presence of the H274Y mutation that confers resistance to oseltamivir (Table 5). NY used dideoxy sequencing of the NA gene for H274Y detection, while WI used pyrosequencing of the H274Y region. Of the samples tested, 91 (39.5%) were typed and/or subtyped as A/H1 virus, 92 (40.4%) as A/H3 virus, and 45 (19.7%) as influenza B virus. While 14.3% of the A/H1 samples possessed the H274Y mutation, all of the A/H3 and B virus samples were found to have a wild-type NA sequence. All samples resistant to oseltamivir were susceptible to adamantanes.

DISCUSSION

In the United States, the rapid increase in prevalence of adamantane resistance in A/H3N2 viruses during the 2005-2006 season, the sudden emergence of oseltamivir resistance in A/H1N1 viruses during the 2007-2008 season, and the presence of oseltamivir resistance in nearly all A/H1 viruses tested in the first part of the 2008-2009 season (8) clearly indicate the need to monitor antiviral resistance in circulating influenza viruses, to ensure that the few effective antiviral agents available are used to the greatest advantage. In NY and WI, adamantane

resistance in A/H3N2 virus increased from 82.7% in the 2006-2007 season to 100% in the 2007-2008 influenza season. However, adamantane resistance in A/H1N1 virus in NY and WI appears to be increasing at a lower rate, from 0% detected in the 2006-2007 season to 1.2% detected in the 2007-2008 season. Nationwide, in the 2007-2008 influenza season, the CDC reported adamantane resistance rates of 99.6% for A/H3N2 virus and 11.1% for A/H1N1 virus; the divergence of our percentages are an indication that regional variations in resistance rates can be substantial. Although the methods described here are usually not rapid enough to facilitate decisions on individual patient treatment, they can influence decision making downstream. Once it has been determined that a susceptible A/H1N1 virus is present, adamantanes can be employed for effective prophylactic and medical treatment of possible patient contacts, especially when the virus strain is oseltamivir resistant. This strategy has particular potential value in residential care centers or other group-living facilities with "at-risk" populations. With the introduction of newer, more rapid, subtype-specific influenza "point-of-care" tests, the ability to empirically determine proper antiviral use will depend heavily on the regional antiviral resistance rates, which are obtained from state surveillance programs.

Pyrosequencing was used to screen specimens for adamantane-resistant influenza A viruses. Pyrosequencing is a relatively new method suitable for rapid acquisition of short reads (100 to 200 bp) of genomic sequence, so as to identify the presence of known mutations. Because the adamantane resistance mutations are clustered in a short span of the influenza M2 gene sequence (see Fig. S1 in the supplemental material), pyrosequencing is an appropriate tool for rapid analysis. Using newly selected data sets, the NY laboratory used a modification of a previously published pyrosequencing method for this genomic region, enabling sequencing of the influenza virus in both directions, as a further confirmatory measure. Additionally, with the design of the new M2 gene dideoxy sequencing assay, we were able to directly compare the two methods. The consistency in results between the pyrosequencing and dideoxy sequencing assays served to validate the pyrosequencing methodology. Occasionally, poor pyrosequencing results were identified by the PyroMark ID software or during postrun analysis. These included spurious peaks, added or deleted bases in homopolymer regions, and wide or double peaks; such errors tend to arise from an overloading of starting material in the pyrosequencing reaction. Reduction of the biotinylated PCR product starting amount to 15 μ l usually corrected the problem.

Results also confirmed that pyrosequencing of extracts from fresh (unfrozen) primary specimens during routine influenza surveillance can be a reliable method for the rapid detection of adamantane and NAI resistance in influenza A viruses. When a primary specimen test results in a pyrosequencing quality rating of "fail," the test should be performed on the corresponding virus culture isolate if available. Overall, we could be reasonably confident that samples with C_t values in the high 20s, or below, in the initial influenza A real-time RT-PCR screening assay would be accurately sequenced with the pyrosequencing method. The cutoff value of 26 was chosen to ensure that a high percentage of the samples tested with pyrosequencing would yield usable sequence. In a public health setting, testing the majority of the samples will often provide

sufficient information for assessment of an outbreak, or evaluation of resistance in a state or region. The cutoff value was included as a possible guideline for obtaining information from the majority of the samples in a short time with minimal unusable results. While bidirectional pyrosequencing is not a necessity in a surveillance system, it does give increased confidence in results, especially if they are to be reported clinically. Also, for samples for which no sequence can be obtained in one direction, the other direction may give acceptable data. In fact, the reverse pyrosequencing method was slightly more reliable in this regard than the originally published forward method (data not shown).

Pyrosequencing was also used to screen specimens for oseltamivir-resistant A/H1N1 viruses. Because the H274Y mutation had previously been detected and was well characterized (9, 17), primers for pyrosequencing of this region (11) were used for rapid screening to detect this mutation. To monitor for other NAI resistance mutations that could be present in currently circulating influenza A/H1N1, A/H3N2, and B virus strains, primers were developed in NY to allow dideoxy sequencing of the NA gene. This method can be more informative than pyrosequencing, since it generates bidirectional sequence from nearly the entire gene, using two sequencing reactions per direction. Unfortunately, sample extracts in some cases had been frozen and thawed up to three times, promoting RNA degradation; this is a factor that can particularly affect influenza B virus, which is more sensitive to repeated freezing and thawing (J.M.L., unpublished observations). Six point mutations conferring amino acid changes, in addition to the H274Y mutation, were detected with this method. Initial testing found that none inhibited NA (data not shown), and further work on these mutations is ongoing. However, given the current specific interest in the circulating influenza A virus oseltamivir-resistant H274Y viruses, any combination of the A/N1 forward and reverse primers in Table 2 can be used in a single assay to detect the H274Y mutation, which is situated in an area common to all primer set amplicons.

In the early part of the 2007-2008 influenza season, routine monitoring of antiviral resistance resulted in the identification of a number of oseltamivir-resistant A/H1N1 viruses. Continued screening during the season confirmed that all oseltamivir-resistant viruses had an NA gene mutation that results in the H274Y amino acid change, and all were subtype A/H1N1. For the 2007-2008 season, the CDC reported that 10.2% of A/H1N1 virus surveillance strains contained the oseltamivir resistance mutation. For NY and WI, the respective rates were 11.1 and 17.4% of A/H1N1 virus-positive specimens. The rapid emergence of oseltamivir resistance in this subtype, from less than 1% to more than 10%, suggests that an increasingly large proportion of A/H1N1 viruses will not be treatable with oseltamivir and that rapid and reliable methods for determining the antiviral resistance status of influenza viruses are crucial. Initial data for the 2008-2009 influenza season indicate that nearly all A/H1 viruses have the H274Y mutation and are oseltamivir resistant (8).

In addition to the sequencing methods presented here for the detection of resistance mutations, real-time PCR methods sensitive enough to allow detection of the separate, single-base-pair differences that confer resistance to influenza antivirals are needed; several new methods have recently been pub-

lished (6, 14). Real-time PCR methods capable of this type of detection will allow increasing numbers of laboratories to monitor resistance, since the instrumentation necessary is already available in a large number of clinical laboratories. We plan to expand our two-state surveillance network for influenza antiviral resistance detection to include other state public health laboratories that perform resistance testing on influenza-positive specimens and isolates. Such a network is needed to provide region-specific antiviral test results in support of CDC testing efforts and will be important in any potential pandemic situation.

ACKNOWLEDGMENTS

We thank Gino Battaglioli for helpful discussions concerning the manuscript and the Wadsworth Center Molecular Genetics Core for all sequencing of isolates from NY. We also thank Varough Deyde and Larisa Gubareva, at the CDC, for primer sequences used in the H274Y pyrosequencing assay.

This work was supported in part by an appointment to the Emerging Infectious Disease Fellowship Program administered by the Association of Public Health Laboratories and was funded in part by the CDC.

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