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Application of a Seven-Target Pyrosequencing Assay To Improve the Detection of Neuraminidase Inhibitor-Resistant Influenza A(H3N2) Viruses

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National U.S. influenza antiviral surveillance incorporates data generated by neuraminidase (NA) inhibition (NI) testing of isolates supplemented with NA sequence analysis and pyrosequencing analysis of clinical specimens. A lack of established correlates for clinically relevant resistance to NA inhibitors (NAIs) hinders interpretation of NI assay data. Nonetheless, A(H3N2) viruses are commonly monitored for moderately or highly reduced inhibition in the NI assay and/or for the presence of NA markers E119V, R292K, and N294S. In 2012 to 2013, three drug-resistant A(H3N2) viruses were detected by NI assay among isolates (n = 1,424); all showed highly reduced inhibition by oseltamivir and had E119V. In addition, one R292K variant was detected among clinical samples (n = 1,024) by a 3-target pyrosequencing assay. Overall, the frequency of NAI resistance was low (0.16% [4 of 2,448]). To screen for additional NA markers previously identified in viruses from NAI-treated patients, the pyrosequencing assay was modified to include Q136K, I222V, and deletions encompassing residues 245 to 248 (del245-248) and residues 247 to 250 (del247-250). The 7-target pyrosequencing assay detected NA variants carrying E119V, Q136, and del245-248 in an isolate from an oseltamivir-treated patient. Next, this assay was applied to clinical specimens collected from hospitalized patients and submitted for NI testing but failed cell culture propagation. Of the 27 clinical specimens tested, 4 (15%) contained NA changes: R292K (n = 2), E119V (n = 1), and del247-250 (n = 1). Recombinant NAs with del247-250 or del245-248 conferred highly reduced inhibition by oseltamivir, reduced inhibition by zanamivir, and normal inhibition by peramivir and laninamivir. Our results demonstrated the benefits of the 7-target pyrosequencing assay in conducting A(H3N2) antiviral surveillance and testing for clinical care.

Two neuraminidase inhibitors (NAIs), inhaled zanamivir and oral oseltamivir, are currently licensed in the United States for the treatment of influenza A and B virus infections (1). In addition, intravenous peramivir (2) is licensed in Japan, South Korea, and China, and inhaled laninamivir (3) is also licensed in Japan. Monitoring the susceptibility of influenza viruses to NAIs has become an integral part of virological surveillance conducted globally within the WHO Global Influenza Surveillance and Response System (WHO-GISRS) (4). Assessment of influenza virus susceptibility to NAIs is primarily performed using NA inhibition (NI) assays; viruses showing reduced inhibition are further tested using genetic methods such as pyrosequencing (5) and/or Sanger sequence analysis (6) to identify the NA changes (molecular markers) responsible for the increased 50% inhibitory concentrations (IC_{50}). Notably, propagation of contemporary A(H3N2) viruses in tissue cultures such as Madin-Darby canine kidney (MDCK) cells, a prerequisite of the NI assay, may give rise to virus subpopulations with changes in the NA (e.g., D151) that are often absent in matching original clinical samples (7–10). For this reason, pyrosequencing testing is routinely performed on a matching clinical sample (when available) to confirm the presence of the NA marker detected in the isolate.

Culturing of influenza viruses is time-consuming, and the rate of virus recovery depends on many factors (11). Therefore, the pyrosequencing assay was implemented to enhance influenza antiviral surveillance in the United States. The CDC pyrosequencing assay (12) was designed to detect amino acid substitutions in the NA known to emerge after treatment with NAI(s) and to confer (highly) reduced inhibition in the NI assay. In accordance with the guidance provided by the World Health Organization Influenza Antiviral Working Group (WHO-AVWG), these NA markers are H275Y and I223R/K in A(H1N1)pdm09 viruses and E119V, R292K, and N294S in A(H3N2) viruses (13). Since 2009, during each influenza season, a large subset of clinical samples has been designated for testing, without propagation, by means of pyrosequencing. Several state public health laboratories (PHLs) contribute their pyrosequencing results to national surveillance; laboratories lacking pyrosequencing capability submit clinical samples for testing to the designated contract PHL (14). Combined results from NI assay testing of virus isolates and pyrosequencing of un-

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related clinical samples are updated weekly in the CDC FluView report (15) during the influenza season.

The emergence of influenza A(H3N2) viruses carrying E119V and R292K (7, 16–19) and, to a lesser extent, N294S (20) in oseltamivir-treated patients has been reported. Viruses containing E119V are reported to national surveillance as oseltamivir resistant, while those carrying R292K are reported as resistant to oseltamivir and zanamivir. Besides these 3 markers, other NA changes have been reported to affect susceptibility to NAIs. E119I substitution was detected in an influenza A(H3N2) virus isolate from an oseltamivir-treated patient (7); however, the matching clinical sample necessary to confirm the presence of the substitution was unavailable. The I222V substitution, detected in combination with E119V, in a virus recovered from an oseltamivir-treated immunocompromised patient resulted in an ~1,000-fold increase in the IC_{50} for oseltamivir compared to the wild-type virus (19). In addition to substitutions, NA changes comprising 4-amino-acid deletions encompassing either residues 245 to 248 (del245-248) (18, 21, 22) or residues 247 to 250 (del247-250) (23) in A(H3N2) viruses recovered from oseltamivir-treated patients have been reported. The del245-248 mutant in combination with E119V and Q136K (22) was detected in an immunocompromised patient on prolonged therapy with oseltamivir and zanamivir. The Q136K substitution was reported in two field isolates from patients with unknown drug exposure (24); these isolates exhibited reduced inhibition by zanamivir, with 30- and 53-fold increases in the zanamivir IC_{50}, respectively, compared to a wild-type virus.

The 3-target pyrosequencing assay has been successfully applied in detecting the NA molecular markers of drug resistance, E119V, R292K, and N294S, which are recommended by the WHO-AVWG for monitoring influenza antiviral drug susceptibility in influenza A(H3N2) viruses. To increase its scope, the 3-target pyrosequencing assay was modified to allow the detection of four additional markers of drug resistance in A(H3N2) viruses, including Q136K, I222V, del245-248, and del247-250. This report describes the consequent 7-target pyrosequencing assay and its use in antiviral surveillance during the influenza season of 2012 to 2013.

MATERIALS AND METHODS

Viruses and cells. Influenza A(H3N2) virus isolates and clinical specimens collected in the United States between 1 October 2012 and 30 September 2013 were submitted to the U.S. WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza, in Atlanta, GA. Viruses were propagated in MDCK cells (ATCC, Manassas, VA) and antigenically characterized by the hemagglutination inhibition (HI) assay prior to antiviral susceptibility testing.

Neuraminidase inhibitors. Oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was kindly provided by Hoffmann-La Roche (Basel, Switzerland), zanamivir by GlaxoSmithKline (Uxbridge, United Kingdom), peramivir by BioCryst Pharmaceuticals (Birmingham, AL), and laninamivir (R-125489) by Biota (Melbourne, Australia).

Neuraminidase inhibition assay. The susceptibility of virus isolates to the four NAIs was assessed in the NI fluorescence assay, using an NA-Fluor influenza neuraminidase assay kit (Applied Biosystems, Foster City, CA) with modifications to the manufacturer's protocol (25). Influenza A(H3N2) reference viruses from the CDC panel, A/Washington/01/2007 wild type and /Texas/12/2007 E119V, were included in the assay. The drug concentration required to inhibit NA activity by 50% (IC_{50}) was determined using JASPR v1.2 curve-fitting software (CDC, Atlanta, GA). Fold changes in IC_{50} were determined by comparing the IC_{50}s of test viruses with that of the drug-susceptible wild-type reference virus, A/Washington/01/2007 (H3N2). Fold changes were interpreted according to classification criteria recommended by the WHO-AVWG (26). Viruses with a $<$10-fold change in the IC_{50} value were characterized as exhibiting normal inhibition by the corresponding NAI, while those with a 10- to 100-fold change and a $>$100-fold change were characterized as exhibiting reduced and highly reduced inhibition, respectively.

Pyrosequencing assay. A 7-target pyrosequencing assay was used to detect mutations at residues 119, 136, 222, 292, and 294 of the NA, as well as 4-amino-acid deletions at residues 245 to 248 and residues 247 to 250. The assay was performed using a PyroMark PSQ96 MA platform (Qiagen, Valencia, CA) as previously described (12). Primers for reverse transcription-PCR (RT-PCR) and sequencing were designed using Pyrosequencing Assay Design software (Qiagen, Valencia, CA) and are listed in Table S1 in the supplemental material. The pyrosequencing assay for detecting the 4-amino-acid-deletion variants, del245-248 and del247-250, utilized a customized nucleotide dispensation, AATGCAATGCATGCATGCAATG CTAATACTA.

Sanger sequencing. Sanger sequencing of whole-NA genes was performed as previously described (27). Primers for RT-PCR and sequencing are listed in Table S2 in the supplemental material. The NA sequence data were deposited to the Global Initiative on Sharing All Influenza Data (GISAID). GISAID accession numbers are provided in Table 1.

recNA proteins. Mutant recombinant NA (recNA) proteins were prepared on the A/Perth/16/2009 framework as previously described (8). Additionally, two subsequent NA variants (del245-248 and del247-250) were generated from a wild-type pIEx-4-NA clone using a QuikChange Lightning site-directed mutagenesis kit (Agilent, CA). All constructs were transiently transfected into sf9 cells (EMD Millipore, MA) using Cellfectin II transfection reagent (Life Technologies, NY), according to the manufacturer's protocols. At 5 days posttransfection, supernatants containing secreted recNA were harvested and clarified and the recNA was quantified by Western blot analysis, using baculovirus-expressed and purified A/Perth/16/2009 recNA protein as a control.

RESULTS

During the 2012 to 2013 influenza season, 1,744 viruses submitted for U.S. national virological surveillance were inoculated in MDCK cells for virus isolation, 1,424 (82%) of which grew successfully. All propagated viruses were tested in the NI fluorescence assay, and all were susceptible to FDA-approved oseltamivir and zanamivir, with the exception of three (0.2%) which exhibited highly reduced inhibition by oseltamivir ($>$100-fold increase in IC_{50}) (Table 1). Each of these viruses, namely, A/Massachusetts/07/2013 (passage M1/C2), A/Arkansas/13/2013 (passage C1), and A/Texas/08/2013 (passage C2), possessed the E119V NA substitution which is associated with oseltamivir resistance. A/Massachusetts/07/2013 (M1/C2) contained a predominantly E119V NA variant (90%), and since no matching clinical sample was available, pyrosequencing testing was done on the isolate received (passage M1), which showed the presence of E119V (70%). This marker was also confirmed in the original clinical specimens of A/Arkansas/13/2013 (75%) and A/Texas/08/2013 (53%). Besides E119V, both of the latter clinical specimens contained a virus population with R292K, a marker of resistance to oseltamivir and zanamivir, at proportions of 25% and 42%, respectively. The discrepancy observed between the pyrosequencing data for clinical samples and the data for virus isolates of A/Arkansas/13/2013 and A/Texas/08/2013 could be a consequence of the reduced replicative fitness of R292K viruses (28).

A subset of influenza viruses ($n = 439$), including the three virus isolates carrying E119V, was also assessed for susceptibility to the NAIs peramivir and laninamivir; all tested viruses

TABLE 1 NAI susceptibility of influenza A(H3N2) virus isolates assessed in the NI fluorescence assay, coupled with NA sequencing analysis

Virus	Passage	NA change (frequency of variants, %)[a]			Mean IC_{50} ± SD,[c] nM (fold change)[b]			
		E119	Q136	R292	Zanamivir	Oseltamivir	Peramivir	Laninamivir
A/Arkansas/13/2013	Clinical	E119V (75)	—[d]	R292K (25)	—	—	—	—
	C1[e]	E119V (100)	—	R292	0.33 ± 0.02 (1)	35.55 ± 2.84 (395)	0.11 ± 0.00 (1)	0.39 ± 0.01 (1)
A/Texas/08/2013	Clinical	E119V (53)	—	R292K (42)	—	—	—	—
	C2[f]	E119V (96)	—	R292	0.29 ± 0.03(1)	29.34 ± 6.30 (326)	0.11 ± 0.00 (1)	0.57 ± 0.00 (2)
A/Massachusetts/07/2013	M1[g]	E119V (70)	Q136	R292	—	—	—	—
	M1/C2[h]	E119V (90)	Q136K (23)	R292	1.32 ± 0.00 (6)	45.44 ± 0.00 (505)	0.37 ± 0.00 (4)	0.99 ± 0.00 (4)
A/Massachusetts/07/2013 clone 1	M1/S1[i]	E119V (100)	Q136	R292	0.44 ± 0.10 (2)	37.33 ± 10.4 (415)	0.14 ± 0.08 (2)	0.40 ± 0.01 (1)
A/Massachusetts/07/2013 clone 2, del245-248	M1/S1	E119	Q136	R292	2.78 ± 0.41 (12)	21.70 ± 3.59 (241)	0.37 ± 0.17 (4)	0.40 ± 0.02 (1)
Reference viruses								
A/Washington/01/2007, oseltamivir susceptible		E119	—	R292	0.24 ± 0.02 (1)	0.09 ± 0.02 (1)	0.09 ± 0.01 (1)	0.28 ± 0.02 (1)
A/Texas/12/2007, oseltamivir resistant		E119V	—	R292	0.39 ± 0.03 (2)	47.81 ± 4.46 (531)	0.10 ± 0.02 (1)	0.33 ± 0.05 (1)

[a] Proportion of NA variants versus wild type, determined using single nucleotide polymorphism (SNP) pyrosequencing analysis. GISAID accession numbers for the NA gene: EPI484515, EPI436296, and EPI447137.
[b] Fold increase based on comparison to the respective IC_{50}s of the reference virus, A/Washington/01/2007.
[c] Data are based on the results of at least three independent experiments. A/Massachusetts/07/2013 (passage M1/C2) was tested only once by NI assay due to insufficient sample volume.
[d] —, test not performed.
[e] C1, the original clinical specimen was passaged once in Madin-Darby canine kidney (MDCK) cells at the CDC reference laboratory.
[f] C2, the original clinical specimen was passaged twice in MDCK cells at the CDC reference laboratory.
[g] M1, the original clinical specimen was passaged once in rhesus monkey kidney (RMK) cells by the submitting laboratory (prior to submission to CDC).
[h] M1/C2, the original clinical specimen was passaged once in RMK cells by submitting laboratory and then (M1) passaged twice in MDCK cells at CDC.
[i] M1/S1, the original clinical specimen was passaged once in RMK cells by the submitting laboratory; then, at CDC, the M1 was plaque purified and the clones were passaged once in MDCK-SIAT1 cells.

exhibited normal inhibition by both drugs (<10-fold increase in IC_{50}). Two virus isolates that exhibited highly reduced inhibition by oseltamivir, A/Arkansas/13/2013 (passage C1) and A/Texas/08/2013 (passage C2), showed normal inhibition by zanamivir and peramivir and laninamivir NI assay profiles characteristic of E119V NA variants. Notably, A/Massachusetts/07/2013 (passage M1/C2) exhibited slightly (3- to 5-fold) elevated IC_{50}s for zanamivir, peramivir, and laninamivir, which prompted additional investigations. Since the 7-target pyrosequencing assay was developed by this time, it was used to analyze the A/Massachusetts/07/2013 isolate (passage M1/C2). The assay revealed the presence of a population carrying Q136K (23%), in addition to the E119V substitution (90%). The Q136K substitution was, however, not detected in the isolate received (M1 passage) by either pyrosequencing or sequencing analysis, suggesting that the MDCK cells selected for this NA variant.

To further characterize the virus populations in A/Massachusetts/07/2013, the isolate received (M1 passage) was plaque purified in MDCK-SIAT1 cells. A total of 20 plaque-purified clones were randomly picked, propagated once in MDCK-SIAT1 cells (to passage M1/S1), and analyzed using the 7-target pyrosequencing assay. Of the 20 plaque-purified viruses tested, 12 possessed an E119V substitution, and one had the del245-248 deletion. Conventional Sanger sequence analysis confirmed the presence of del245-248 in the latter virus. Two plaque-purified viruses, with E119V and del245-248, respectively, were tested in the NI fluorescence assay. The virus with E119V had an NI assay profile characteristic of other E119V variants (Table 1); the one with del245-248 also exhibited highly reduced inhibition by oseltamivir, reduced inhibition by zanamivir, and normal inhibition by the other two NAIs. These results were consistent with those previously reported for this NA variant (21, 22).

In 2012 to 2013, 320 (18%) of the A(H3N2) clinical specimens submitted to CDC for antigenic and antiviral characterization failed to propagate in cell culture; therefore, NI assay testing could not be performed on these samples. Based on information provided in the virus submission forms, 27 of these specimens were collected from patients at high risk for developing drug resistance, including those (deceased or not deceased) with histories of hospitalization and/or oseltamivir exposure. These 27 clinical specimens were tested using the 7-target pyrosequencing assay, and four (14.8%) contained changes in the NA (Table 2). Two specimens, A/Minnesota/21/2013 and A/Nebraska/20/2013, had R292K at 100% and 37%, respectively, of each population. One specimen, A/Arkansas/35/2013, contained E119V (100%), while A/Arkansas/36/2013 had del247-250 (100%). The pyrogram for the NA with this 4-amino-acid deletion is shown in Fig. S1 in the supplemental material. To our knowledge, the del247-250 mutant has previously been reported only once in an influenza A(H3N2) virus collected from an oseltamivir-treated patient; however, no

TABLE 2 NA changes detected by 7-target pyrosequencing testing of influenza A(H3N2) clinical specimens from which virus was not recovered

Virus	Subject age (yrs)	Subject sex[a]	Subject history[b]	NA change (frequency of variant, %)
A/Arkansas/35/2013	72	M	Suspected drug resistance	E119V (100)
A/Arkansas/36/2013	66	M	Suspected drug resistance	del247-250 (100)
A/Minnesota/21/2013	86	M	Suspected drug resistance	R292K (100)
A/Nebraska/20/2013	1	M	Deceased	R292K (37)

[a] M, male.
[b] Preexisting medical conditions, immune status, treatment, patient outcome, etc.

virus isolate was obtained and therefore no NI testing could be performed (23).

To ascertain the effects of del247-250 on inhibition of the NA enzyme activity by NAIs, recombinant NAs (recNAs) with and without del247-250 were generated on the A/Perth/16/2009 NA framework. In addition, a recNA carrying the del245-248 deletion was also generated. RecNA proteins were tested in the NI assay against four NAIs (Table 3). Compared to the wild type, the recNA with del247-250 showed ~235-fold and 17-fold increases in IC_{50}s for oseltamivir and zanamivir, respectively, while the peramivir IC_{50} was only slightly affected (5-fold) and the laninamivir IC_{50} was unaffected (Table 3). The recNA with del245-248 exhibited IC_{50}s comparable to those of the virus isolate with del245-248 (Table 1), and its IC_{50} profile was also similar to that of the recNA with del247-250 (Table 3). Overall, both deletion mutations resulted in highly reduced inhibition by oseltamivir, reduced inhibition by zanamivir, and normal inhibition by the other two NAIs. To determine whether the 4-amino-acid deletions would affect NA activity and thus interfere with virus recovery, the respective recNA preparations were normalized to protein content, and their NA activity was assessed using MUNANA [2′-(4-methylumbelliferyl)-alpha-D-*N*-acetylneuraminic acid] substrate. The two deletions caused similar (65% to 68%) reductions in NA activity (Table 3).

DISCUSSION

The majority of viruses submitted for U.S. national virological surveillance are collected from untreated patients or just prior to initiation of drug treatment. PHLs and the CDC also receive requests for antiviral testing for clinical management of a particular patient or an outbreak control (in nursing care facilities). Samples received for such testing are typically collected from patients following initiation of antiviral treatment or prophylaxis. In such instances, the pyrosequencing assay is used to test clinical samples to identify molecular markers of resistance to a particular antiviral drug and to facilitate decisions on clinical management and outbreak control. Pyrosequencing is also utilized to enhance national antiviral surveillance by testing clinical samples that are not submitted for comprehensive virological characterization. Testing for both diagnostic and surveillance purposes in influenza A(H3N2) viruses has been limited to 3 molecular markers associated with resistance to an NAI(s), namely, substitutions at residues 119, 292, and 294, as outlined in the WHO-AVWG guidance (13).

Pyrosequencing using the PyroMark platform offers many advantages over conventional Sanger sequencing (29). It was instrumental in detecting molecular markers of resistance to M2 blockers, since all known markers of resistance to this class of drugs in all influenza A subtypes could be detected using a single pyrosequencing reaction (30). However, this is not the case with NAIs, whose molecular markers of resistance differ among subtypes and by NAI. Moreover, markers of NAI resistance are spread widely apart along the NA, which necessitates running several pyrosequencing reactions using target-specific amplicons. For example, in this study, performing the 7-target pyrosequencing assay required generation of three RT-PCR amplicons and running five pyrosequencing reactions. The use of newer sequencing technologies such as the MiSeq system (Illumina, San Diego, CA) can be helpful in detecting a variety of NA variants in clinical specimens (22). However, these technologies are currently used mainly for research purposes, although it is plausible that they will provide a Clinical Laboratory Improvement Amendments (CLIA)-certified method for detecting drug resistance in influenza viruses by surveillance and clinical laboratories in the future.

In the present study, the 7-target assay was applied to 27 clinical specimens from which infectious virus was not recovered. This resulted in detection of four resistant viruses that would have remained undetected under our current testing algorithm. Moreover, one of these resistant viruses had a 4-amino-acid deletion in the NA, del247-250, which was undetectable by the 3-target pyrosequencing assay. Developing tools for detecting such deletion mutations may facilitate their identification as well as their inclusion in the list of mutations recommended by the WHO for monitoring influenza antiviral drug susceptibility (13).

Recombinant protein technology enabled the generation of recNAs that contributed to understanding the effects of the 4-ami-

TABLE 3 Characterization of recombinant neuraminidases in the NI fluorescence assay

NA change	Mean IC_{50} ± SD,[a] nM (fold change)[b]				Avg NA activity, % (fold change)[c]
	Zanamivir	Oseltamivir	Peramivir	Laninamivir	
del245-248	3.48 ± 0.48 (21)	22.29 ± 1.43 (222)	0.35 ± 0.04 (5)	0.42 ± 0.06 (1)	68 (2)
del247-250	2.74 ± 0.31 (17)	23.56 ± 3.92 (235)	0.41 ± 0.02 (5)	0.40 ± 0.07 (1)	65 (2)
A/Perth/16/2009	0.16 ± 0.05	0.10 ± 0.04 (1)	0.07 ± 0.03 (1)	0.23 ± 0.02 (1)	100 (1)

[a] Data are based on the results of at least three independent experiments.
[b] Fold increase based on comparison to the respective IC_{50}s of the recombinant NA with no deletion (wild type).
[c] Fold decrease based on comparison to the NA activity of recombinant NA with no deletion (wild type).

no-acid deletions on drug susceptibility and NA activity. In this study, del245-248 and del247-250 reduced NA activity (by 65% to 68%), while the del245-248 variant had no apparent effect on NA activity in a previous study (21). Similarly to the present study, del245-248 reported in A(H3N2) viruses (18, 21) resulted in an ~200-fold increase in the oseltamivir IC_{50} and no apparent effect on the zanamivir IC_{50}. Unlike the E119V and R292K substitutions, whose effects on NAI susceptibility are well characterized (7, 16–19), the exact mechanisms by which these deletion mutants affect NAI susceptibility remain unclear. A recent report (22) suggests that, since A246 is directly involved in formation of the hydrophobic pocket adjacent to the NA enzyme cavity, conformational changes at the surface level caused by del245-248 could disturb the hydrophobic side pocket that accommodates the glycerol side chain of sialic acid and zanamivir, which may affect NAI binding. Further studies are needed to confirm this effect for the del247-250 NA variant.

On the basis of available epidemiological information, all resistant viruses detected in this study were from hospitalized and/or treated patients. The four NAI-resistant virus variants detected in clinical samples by 7-target pyrosequencing were from patients (3 elderly and 1 infant) at high risk for influenza complications, for whom antiviral treatment is typically recommended (1). Specimens from the elderly patients were submitted specifically for antiviral surveillance, which typically occurs when drug resistance is suspected, suggesting that the patients were treated with influenza antivirals. The pediatric patient was deceased, suggesting hospitalization and probable antiviral treatment. Among the NA variants detected by the NI assay, A/Massachusetts/07/2013, with subpopulations of del245-248, E119V, and Q136K, was collected from an immunocompromised patient on prolonged oseltamivir treatment, as was A/Arkansas/13/2013 with E119V. The E119V variant, A/Texas/08/2013, was from a deceased pediatric patient; no information on oseltamivir exposure was available, but the patient was most likely on antiviral treatment during hospitalization.

Since selection of samples for antiviral testing is currently based on information provided in the submission forms, which often lack data on drug exposure, it is prudent to extend the 7-target pyrosequencing testing to samples collected from hospitalized patients and fatal cases, regardless of the availability of information on drug exposure. By using this algorithm, we will not only count resistance cases which would otherwise be unreported but will also gain valuable information regarding NA variants which may not propagate well in cell cultures. Timely detection of resistance in unpropagated viruses by pyrosequencing will also facilitate epidemiological investigations regarding antiviral exposure.

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