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Analysis of influenza viruses from patients clinically suspected of infection with an oseltamivir resistant virus during the 2009 pandemic in the United States

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ABSTRACT

During the 2009 influenza pandemic, the Centers for Disease Control and Prevention provided antiviral susceptibility testing for patients infected with suspected drug-resistant viruses. Specimens from 72 patients admitted to an intensive care unit or with a severe immunocompromising condition, who failed to clinically improve after oseltamivir treatment, were accepted for testing. Respiratory specimens were tested for the presence of the oseltamivir resistance-conferring H275Y substitution in the neuraminidase (NA) by pyrosequencing. Virus isolates propagated in MDCK cells were tested in phenotypic NA inhibition (NI) assays using licensed NA inhibitors (NAIs), zanamivir and oseltamivir, and investigational NAIs, peramivir and laninamivir. Conventional sequencing and plaque purification were conducted on a subset of viruses. Pyrosequencing data were obtained for 87 specimens collected from 58 of the 72 (81%) patients. Of all patients, 27 (38%) had at least one specimen in which H275Y was detected. Analysis of sequential samples from nine patients revealed intra-treatment emergence of H275Y variant and a shift from wild-type-to-H275Y in quasispecies during oseltamivir therapy. A shift in the H275Y proportion was observed as a result of virus propagation in MDCK cells. Overall, the NI method was less sensitive than pyrosequencing in detecting the presence of H275Y variants in virus isolates. Using the NI method, isolates containing H275Y variant at $\geqslant$ 50% exhibited resistance to oseltamivir and peramivir, but retained full susceptibility to zanamivir. H275Y viruses recovered from two patients had an additional substitution I223K or I223R that conferred a 38–52- and 33–97-fold enhancement in oseltamivir- and peramivir-resistance, respectively. These viruses also showed decreased susceptibility to zanamivir and laninamivir. These data suggest that pyrosequencing is a powerful tool for timely detection of NAI resistant viruses and that NI assays are needed for comprehensive testing to detect novel resistance substitutions.
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1. Introduction

The emergence of neuraminidase (NA) inhibitor (NAI) resistance in influenza viruses has both clinical and public health relevance. Circulation of oseltamivir-resistant viruses in the community limits antiviral treatment options, and the development of oseltamivir resistance while on therapy requires prompt identification of resistance to optimize clinical management. Resistance to NAIs can be detected by functional (a substitute for a phenotypic assay which is unavailable for this class of drugs) and genotypic assays. Functional assays detect susceptibility to specific NAIs and report a 50% inhibitory concentration (IC_{50}) for each drug that is interpreted based on laboratory standards. Genotypic assays detect molecular markers that are associated with drug resistance. Currently, genotypic assays for detection of H275Y substitution, a marker associated with oseltamivir resistance in the NA of 2009 pandemic influenza A(H1N1) viruses (H1N1pdm09), are available at a number of commercial, clinical, and public health laboratories in the US. The availability of functional assays is currently limited to the Collaborating Centers of the World Health Organization and some academic and public health laboratories.

During the 2009 pandemic, all but very few H1N1pdm09 viruses were resistant to adamantanes and susceptible to the licensed NAIs, oseltamivir and zanamivir (Bautista et al., 2010; Gubareva et al., 2010). However, prior to the emergence of H1N1pdm09 viruses, oseltamivir-resistant seasonal influenza A(H1N1) viruses circulated in high prevalence in the community (Dharan et al., 2009; Meijer

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et al., 2009), and the emergence of oseltamivir resistance during oseltamivir use had been documented, especially among persons with prolonged virus shedding (Hayden, 2009). During the 2009 H1N1 pandemic, enhanced national surveillance for antiviral resistance detected a low prevalence of oseltamivir-resistant H1N1 pdm09 viruses in the US (Graitcer et al., 2011). In addition, the Centers for Disease Control and Prevention (CDC) tested specimens from patients with clinical suspicion of infection with oseltamivir-resistant H1N1pdm09 viruses for the associated H275Y marker as a service for clinical providers. Results from pyrosequencing were reported to the specimen submitters within 48 h of specimen receipt. Here we summarize our results from testing specimens from patients clinically suspected of oseltamivir-resistant H1N1pdm09 infection from October 2009 until June 2010, and compare molecular and functional antiviral resistance results to help provide future guidance and testing algorithms.

2. Materials and methods

2.1. Specimens

Since October 2009, CDC has provided testing for clinical respiratory specimens from patients with H1N1pdm09 virus infection who were clinically suspected to have an oseltamivir-resistant virus infection. Testing was prioritized for patients, with either immunocompromising conditions or those admitted to the intensive care unit (ICU), who had received at least 5 days of oseltamivir treatment with no signs of clinical improvement. However, specimens from any patient suspected of infection with a resistant virus were also accepted, including specimens from patients who developed H1N1pdm09 infections after oseltamivir chemoprophylaxis. All oseltamivir-resistant viruses detected from these patients were reported in national or international summary reports (CDC, 2011; WHO, 2011). Four patients were part of an institutional outbreak investigation (Chen et al., 2011). Demographic, treatment, and underlying medical condition information was submitted with clinical specimens.

2.2. Pyrosequencing

Viral RNA was extracted directly from clinical specimens or grown H1N1pdm09 virus isolates. RT-PCR amplification of viral genes was performed as described previously (Deyde et al., 2010). Pyrosequencing was carried out using the Pyromark Q96 ID software (Qiagen, Valencia, CA), which has two analysis modes, sequencing (SQA) and single nucleotide polymorphism (SNP). SQA was utilized for the 275 and 223 (N1 numbering) amino acid residues in the NA of H1N1pdm09 viruses, and SNP was used to determine the percent composition of variants with H or Y at residue275.Pyrosequencing was performed using target-specific primers as outlined in Clinical Laboratory Improvement Amendments (CLIA)-certified CDC protocols, as described previously (Deyde et al., 2010). Results were recorded as wildtype (H275; $\geqslant$90% wildtype variant), H275Y variant (H275Y; $\geqslant$90% H275Y variant), or mixture (275H/Y; 10% < H275Y variant <90%) (Lackenby et al., 2008).

2.3. Virus isolation

Influenza virus isolation was performed using MDCK-ATCC cells (ATCC, Manassas, VA) according to a standard procedure (Nguyen et al., 2010b). Clinical specimens were grown for one to two passages in order to isolate virus with sufficient NA activity to perform neuraminidase inhibition (NI) assays.

2.4. Neuraminidase inhibition assays

Chemiluminescent (CL) and fluorescent (FL) NI assays were performed on virus isolates. These assays determine IC_{50} value, a concentration (nM) of inhibitor needed to reduce the NA activity by 50% for each drug. Oseltamivir carboxylate, the active metabolite of oseltamivir (Hoffman-La Roche, Basel, Switzerland), zanamivir (GlaxoSmithKline, Uxbridge, UK), and investigational inhibitors, peramivir (BioCryst Pharmaceuticals, Birmingham, AL) and laninamivir (compound R-125489; Daiichi Sankyo, Tokyo, Japan, and Biota, Melbourne, Australia), were used. For simplicity, oseltamivir carboxylate is abbreviated as oseltamivir throughout the text. IC_{50} value was calculated using JASPR curve-fitting software (Okomo-Adhiambo et al., 2010b). Curve-fitting in JASPR was based on the equation: $V = V_{max} * (1 - ([I]/(Ki + [I])))$, where V_{max} is the maximum rate, $[I]$ is the inhibitor concentration, V is the response being inhibited, and Ki is IC_{50} for the inhibition curve. Microsoft Office Access and Excel 2007 were used for data analyses. Drug resistance or reduced susceptibility was defined based on the elevation (fold change) of IC_{50} values of test viruses compared to IC_{50} values of the wildtype reference virus. IC_{50} values were calculated without substrate volumes for FL assays as previously described (Gubareva et al., 2010; Okomo-Adhiambo et al., 2010b).

2.5. Sanger sequencing

Sequence analysis of full-length NA gene was carried out as previously described (Okomo-Adhiambo et al., 2010a) on all viruses with an abnormal FL or CL assay result. Sequences were generated by the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) and assembled using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI).

2.6. Plaque purification

Variants present in mixed virus infections, or quasispecies, from two patients A and B were cloned by plaque purification in MDCK cells, according to procedures described previously (Okomo-Adhiambo et al., 2010a). A total of 10 plaques for the viruses from each patient were randomly picked and analyzed. NA genes of the plaques were sequenced using Sanger and pyrosequencing. Assessment of drug susceptibility of the plaques was performed using CL assay.

3. Results

3.1. Detection of the H275Y marker of oseltamivir resistance

From October 2009 to June 2010, 105 specimens collected from 72 patients from 29 states were submitted to CDC for antiviral susceptibility testing for clinical care purposes. H275Y variant was detected by pyrosequencing in 50 specimens collected from 27 (38%) patients either as dominant genotype (H275Y) or as mixture with the wildtype (275H/Y) (Table 1). Overall, among patients with available information, H275Y or 275H/Y variants were detected by pyrosequencing in 24 (73%) of 33 patients with an immunocompromising condition, and in one (3%) of 33 patients admitted to the ICU without an immunocompromising condition. Among 21 patients with available data and oseltamivir use, the interval between oseltamivir initiation and collection of the specimen with H275Y or 275H/Y variant was a median of 17 days (range of 3–64 days); however, 14 patients stopped oseltamivir treatment (median of 12 days, range 2–55 days) prior to collection of the specimen with resistant virus, and for three patients, end dates for oseltamivir treatment were missed. The patient without immunocompromising condition

Table 1
Detection of the oseltamivir resistance-conferring H275Y substitution in 2009 pandemic influenza A(H1N1) viruses by pyrosequencing, United States, October 2009–June 2010.

Pyrosequencing (SQA and SNP) data	No. patients (%) (n = 72)	No. specimens (n = 105)
Wildtype virus (H275)	31 (43)	37
Oseltamivir resistant virus (H275Y)	27 (38)	50
H275Y	12	19
275H/Y[a]	6	6
H275-to-H275Y or to -275H/Y shift[b]	8	21
275H/Y-to-H275Yshift	1	4
Indeterminate result	14 (19)	18

No. = number.
[a] Mixture of both wildtype (H275) and H275Y virus variants were detected. The assay limit of detection of a minor population in quasispecies is 10% (Lackenby et al., 2008); any variants detected below 10% were not counted.
[b] Patients with multiple specimens where a shift from H275 to H275Y (n = 5), H275 to mixed 275H/Y (n = 1), or H275 to mixed 275H/Y and then to H275Y (n = 2), occurred (see also Table 2).

and oseltamivir-resistant virus infection had the shortest reported interval between oseltamivir initiation and collection of the specimen with H275Y variant (3 days); the shortest interval among immunosuppressed patients with a resistant virus infection was 6 days. Among 18 patients with information on outcome, seven (39%) deaths were reported. Pyrosequencing yielded indeterminate results for specimens from 14 (19%) patients, likely due to low virus content.

In nine immunosuppressed patients with oseltamivir-resistant variant, two or more sequential specimens were available for evaluation (Supplementary data Table 1). In eight immunosuppressed patients, a virus shift from wildtype-to-H275Y or from wildtype-to-275H/Y was observed over time (Tables 1 and 2), and one patient had an 275H/Y-to-H275Y virus shift. The complete replacement of wildtype virus by H275Y variant was detected in sequential specimens collected at a median of 20 days apart (range of 9–35 days).

3.2. Sensitivity of different assays used in assessment of drug susceptibility of virus isolates

A total of 42 virus isolates, recovered from the clinical specimens of 32 patients, were tested with CL assay with oseltamivir, zanamivir, and peramivir (Table 2). Mean oseltamivir IC_{50} values of 275H/Y and H275Y variants were elevated compared to the reference wildtype viruses. 275H/Y mixtures in virus isolates demonstrated lower IC_{50} values compared to H275Y isolates, and some of these mixed virus isolates would not meet laboratory criteria for oseltamivir resistance (e.g. would not be readily detected in CL assay). Of note, two patients had infections with viruses with two amino acid substitutions in the NA: one patient had H275Y and I223R (Nguyen et al., 2010a; Table 2), and the other had mixed 275H/Y and 223I/K variants (Patient B; Tables 3 and 4). The virus with H275Y and I223R exhibited very high, ~16,000- and ~43-fold, increase in oseltamivir IC_{50} values compared to wildtype and H275Y reference viruses, respectively. The virus with mixed 275H/Y and 223I/K exhibited decrease susceptibility to oseltamivir compared to the wildtype viruses (~3- and 34-fold increase in oseltamivir IC_{50} values in CL and FL assay, respectively).

To separate virus variants from a mixture, we performed plaque purification of samples collected from two cases (patients A and B; Table 3). Ten virus clones were randomly picked and their sequences were determined by Sanger sequencing and pyrosequencing. Half of the clones from case A were H275Y and the other half were wildtype, all contained I223 (wildtype). In case B, seven were dual NA mutants, carrying both H275Y and I223K, and the remaining three were wildtype at both residues. When tested in CL assay, the dual mutants exhibited oseltamivir IC_{50} values of approximately ~10,000- and ~38-fold greater than those of the wildtype and H275Y reference viruses, respectively (Table 3).

H275Y substitution also resulted in an increase in peramivir IC_{50} values, which were additionally increased by the presence of either K or R at 223 (Tables 3 and 4). Although H275Y substitution by itself had no effect on susceptibility to the two other NAIs tested, zanamivir and laninamivir, the virus isolates with H275Y and either K or R substitution at 223 exhibited 11–17-fold increase in zanamivir and laninamivir IC_{50} values.

3.3. Selection of virus variants due to propagation in MDCK cells

To determine whether propagation of mixed 275H/Y variants in MDCK cells and in the absence of drug could lead to a shift in the proportion of oseltamivir-resistant variant, we compared pyrosequencing data obtained from clinical specimens and their matching virus isolates (Table 4). An increase in proportion of H275Y variant occurred in three patients after the first passage compared

Table 2
Susceptibility of virus isolates to licensed and investigational neuraminidase inhibitors by the chemiluminescent neuraminidase inhibition assay.

Virus variant	No. of isolates[a]	Mean IC_{50} ± SD(nM) (fold change)[b] [range]			
		Oseltamivir	Zanamivir	Peramivir	Laninamivir
H275, wildtype	17	0.26 ± 0.07 (**1**) [0.18–0.46]	0.31 ± 0.08 (**1**) [0.27–0.46]	0.08 ± 0.02 (**1**) [0.05–0.12]	n/t
275H/Y, mixed	5[c]	23.72 ± 21.76 (**103**) [0.59–48.41]	0.29 ± 0.08 (**1**) [0.23–0.41]	3.91 ± 3.49 (**39**) [0.13–7.66]	n/t
H275Y	18	86.26 ± 30.08 (**375**) [40.78–154.28]	0.36 ± 0.12 (**1**) [0.22–0.74]	8.92 ± 2.41 (**89**) [5.52–15.30]	n/t
H275Y and I223R	2[d]	3750.68 ± 854.98 (**16,307**)	5.04 ± 0.60 (**17**)	897.54 ± 154.58 (**8975**)	3.36 ± 0.42 (**17**)
Reference viruses					
A/California/07/2009 (H275)	–	0.23 ± 0.08 [0.14–0.50]	0.30 ± 0.08 [0.21–0.59]	0.10 ± 0.03 [0.05–0.22]	0.20 ± 0.05
A/Texas/48/2009 (H275Y)	–	72.75 ± 16.99 (**316**) [62.39–119.51]	0.40 ± 0.06 (**1**) [0.26–0.48]	9.29 ± 1.28 (**93**) [6.15–11.45]	0.36 ± 0.07 (**2**)

No., number; n/t, not tested.
[a] Viruses recovered in MDCK cells from 32 patients.
[b] IC_{50}, the concentration (nM) of a drug needed to inhibit 50% of the neuraminidase activity; IC_{50} value of each isolate was calculated based on at least two independent tests conducted in duplicates. Fold change (in bold) was compared to the IC_{50} of the wildtype reference virus, A/California/07/2009 H1N1pdm09.
[c] Virus isolate from one patient contained mixed virus variants at both 275 (H/Y) and 223 (I/K) (see Table 4 and 5, patient B).
[d] Virus isolates recovered from two clinical specimens collected at different time points from the same patient; (Nguyen et al., 2010a); GISAID accession numbers: EPI_ISL_75889 (clinical specimen) and EPI_ISL_76069 (virus isolate); one isolate was tested once in duplicate, and the other was tested three times in duplicates.

Table 3
Drug susceptibility of viral clones in the chemiluminescent neuraminidase inhibition assay.

Patient[a]	Number of clones[b]	Virus variant	Mean IC_{50} ± SD(nM) (fold change)[c]			
			Oseltamivir	Zanamivir	Peramivir	Laninamivir
A	5	H275	0.20 ± 0.03 (**1**)	0.40 ± 0.05 (**2**)	0.11 ± 0.02 (**2**)	n/t
	5	H275Y	70.04 ± 4.78 (**350**)	0.43 ± 0.11 (**2**)	7.65 ± 1.27 (**153**)	n/t
B	3	H275 and I223	0.20 ± 0.03 (**1**)	0.36 ± 0.05 (**2**)	0.10 ± 0.01 (**2**)	0.18 ± 0.05 (**1**)
	7	H275Y and I223 K	2164.30 ± 1241.06 (**10,822**)	2.13 ± 0.27 (**11**)	267.15 ± 43.40 (**5343**)	2.12 ± 0.13 (**11**)
Reference viruses						
A/California/07/2009		H275	0.20	0.20	0.05	0.20 ± 0.05
A/North Carolina/39/2009		H275Y	57.19 (**286**)	0.22 (**1**)	8.14 (**163**)	0.36 ± 0.07 (**2**)

n/t, not tested.
[a] Virus isolates from two patients A and B contained mixtures (H/Y) at 275.
[b] Ten viral clones of each isolate were picked randomly and analyzed by Sanger sequencing and pyrosequencing to determine the neuraminidase sequence at residue 275 and 223.
[c] Mean IC_{50} for individual clones was calculated based on at least two independent tests conducted in duplicates. Fold change (in bold) was compared to the mean IC_{50} of the wildtype reference virus, A/California/07/2009 (H1N1)pdm09.

to the original clinical specimen. In contrast, the proportion of wildtype increased to 11% in patient B after the first passage (data not shown) and became the dominate variant (60%) after second passage (Table 4). Drug susceptibility of the virus isolates from patients A–D, with mixed 275H/Y variants, was assessed in both CL and FL assays against three NAIs. In CL assay, only viruses from patients C and D demonstrated high oseltamivir IC_{50} values indicative of oseltamivir-resistance, whereas viruses from patient A and B, with 49% and 40% H275Y variant, respectively, displayed only marginal elevation in IC_{50} values compared to the wildtype reference viruses. A similar pattern was observed for peramivir in both NI assays. The fold difference in oseltamivir IC_{50} values between wildtype and 275H/Y for all four isolates was much greater in FL assay, which may suggest that this assay is more sensitive in detecting resistance in mixed virus populations (Table 4).

4. Discussion

In this study, a high proportion of oseltamivir resistance was detected among patients with H1N1pdm09 virus infection who were suspected to have developed resistance to oseltamivir during treatment. Majority of the patients had immunocompromising conditions known to prolong virus shedding (Hayden, 2009; Mehta et al., 2010) and promote emergence of drug resistant viruses. This finding is consistent with US surveillance data (Graitcer et al., 2011) and other reports (Bautista et al., 2010; Harvala et al., 2010; Wang et al., 2010; WHO, 2010, 2011; Yang et al., 2010).

Oseltamivir resistance conferred by H275Y substitution in the NA was detected by pyrosequencing in clinical specimens. Follow-up testing of cultured viruses in NI assays identified two viruses with oseltamivir IC_{50} values greater than those typically seen for H275Y variants. One virus carrying an additional substitution I223R in the NA was previously reported (Nguyen et al., 2010a), whereas the other had a I223K change. To our knowledge, this is the first report of dual H275Y-and-I223K substitution in a H1N1pdm09 virus isolated from a patient. Due to the lack of multiple sequential samples from these patients, it remains unknown whether the viruses acquired the H275Y change first, and then the additional change at I223, or whether the change at I223 preceded H275Y. Both viruses exhibited reduced susceptibility to peramivir, zanamivir, and laninamivir. The emergence of H1N1pdm09 viruses with reduced susceptibility to several NAIs is concerning, as antiviral treatment options are limited. It is important to continue monitoring viruses for the presence of variants at position 223, despite the unclear clinical relevance of such substitutions.

Specimens collected from majority (56%) of patients with oseltamivir-resistant virus infection in this study contained admixture of variant and wildtype viruses, a finding similar to previous reports (Yang et al., 2010). Detecting admixtures of wildtype and oseltamivir-resistant H275Y variants in a specimen raises questions related to interpretation of antiviral susceptibility test results for clinical care use. The proportion of variants in virus quasispecies is likely influenced by multiple factors, including the timing between specimen collection and drug administration. Pyrosequencing is well suited for detection of H275Y variants, even when they are present at a low proportion. Detection of H275Y was reported to the specimen submitter when H275Y content in the virus population was at least 10%, the detectable limit of pyrosequencing (Lackenby et al., 2008). Recently developed PCR-based methods allow detection of H275Y presented at an even lower level (1%; Operario et al., 2010); however, the clinical and public health relevance of detecting such minor virus populations is unclear.

As expected, NI assays were found to be less sensitive in detecting H275Y variants in admixtures compared to pyrosequencing. For example, only a 3-fold increase in oseltamivir IC_{50}, a result that would not indicate the presence of the resistant variant, was detected by CL assay for a virus isolate that contained 40% H275Y (Table 4). Moreover, CL assay appeared somewhat less sensitive compared to FL assay in detecting oseltamivir resistance in virus isolates. This observation is consistent with previous findings where FL assay showed better discrimination in IC_{50} values between wildtype and drug resistant variants (Nguyen et al., 2010b). The necessity to propagate virus prior to testing in NI assays can also cause discord between phenotypic and genotypic data obtained for the unpropagated virus. In this study, culturing of 275H/Y specimens led to a shift in the proportion of wildtype versus H275Y (decrease in some instances and increase in the others), findings similar to previous reports (Yang et al., 2010). Nevertheless, the NI assay is necessary for comprehensive antiviral susceptibility testing, since molecular markers for resistance to zanamivir and the investigational NAIs, peramivir and laninamivir, in H1N1pdm09 viruses are currently not well defined.

Since tests performed in this study were offered for clinical purposes, results may be biased due to certain limitations, such as the timing of specimen collection. We detected H275Y variants within an interval of 3 days to 5 weeks since initiation of oseltamivir treatment. However, the timing for specimen collection during or after therapy was not standardized. Consequently, no conclusions can be drawn regarding development of resistance after oseltamivir exposure. Also, it is unknown whether the patients whose specimens were sent for testing are representative of other patients with immunocompromising conditions, thus the high proportion of resistance detected in this study may not be applicable to other patients.

Table 4
Drug susceptibility assessment of virus isolates containing mixtures in two neuraminidase inhibition assays.

Patient	*H*/*Y* ratio, %		Mean IC50 ± SD(nM),(fold change)[a]					
	Clinical specimen	Virus isolate	Chemiluminescent			Fluorescent		
			Oseltamivir	Zanamivir	Peramivir	Oseltamivir	Zanamivir	Peramivir
A	77/23	51/49	0.88 ± 0.08 (**4**)	0.31 ± 0.04 (**2**)	0.30 ± 0.04 (**6**)	25.15 ± 4.30 (**43**)	0.59 ± 0.13 (**1**)	2.01 ± 0.32 (**8**)
B	3/97[b]	60/40[b]	0.59 ± 0.05 (**3**)	0.41 ± 0.04 (**2**)	0.13 ± 0.02 (**3**)	19.91 ± 3.15 (**34**)	0.67 ± 0.12 (**1**)	1.21 ± 0.16 (**5**)
C	43/57	20/80	33.73 ± 2.12 (**169**)	0.23 ± 0.03 (**1**)	6.35 ± 0.73 (**127**)	303.48 ± 31.33 (**514**)	0.53 ± 0.07 (**1**)	31.91 ± 3.22 (**133**)
D	41/59	7/93	76.11 ± 1.58 (**381**)	0.24 ± 0.04 (**1**)	11.29 ± 1.75 (**226**)	451.12 ± 52.04 (**765**)	0.52 ± 0.07 (**1**)	70.25 ± 3.62 (**293**)
Reference viruses								
A/California/07/2009 (H275)	100/0		0.20 ± 0.08	0.20 ± 0.08	0.05 ± 0.03	0.59 ± 0.15	0.47 ± 0.13	0.24 ± 0.08
A/North Carolina/39/2009 (H275Y)	7/93		57.19 ± 3.50 (**286**)	0.22 ± 0.07 (**1**)	8.14 ± 1.54(**163**)	498.26 ± 15.40 (**845**)	0.54 ± 0.15 (**1**)	64.56 ± 4.54 (**269**)

n/t, not tested.
[a] Mean IC_{50} was calculated based on at least two independent tests conducted in duplicates. Fold change (in bold) was compared to the IC_{50} of the wildtype reference virus, A/California/07/2009 H1N1pdm09.
[b] Also contained the I223K substitution in the neuraminidase (GISAID access number pending).

Interpretation of antiviral resistance test results requires knowledge of current laboratory methods and their limitations. Optimal testing methods vary depending on virus type/subtype and test drug. Thus, interpretation of antiviral susceptibility testing for clinical purposes is complicated, and results may also differ by laboratory. Combination of functional and genotypic assays represents an optimal testing approach and would be most informative in the event of an emerging novel drug resistant variant or novel virus.

5. 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.

6. Potential conflict of interest

The authors declare that there are no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2012.01.006.

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