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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.

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