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Host cell selection of influenza neuraminidase variants: Implications for drug resistance monitoring in A(H1N1) viruses☆

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ABSTRACT

The neuraminidase inhibitors (NAIs), oseltamivir and zanamivir, are essential for treatment and prevention of influenza A and B infections. Oseltamivir resistance among influenza A (H1N1) viruses rapidly emerged and spread globally during the 2007–2008 and 2008–2009 influenza seasons. Approximately 20% and 90% of viruses tested for NAI susceptibility at CDC during these seasons, respectively, were resistant to oseltamivir (IC_{50} ~100–3000 time > those of sensitive viruses), based on the chemiluminescent NA inhibition assay. Pyrosequencing analysis confirmed H274Y mutation (H275Y in N1 numbering) in the neuraminidase (NA) gene of oseltamivir-resistant viruses. Full NA sequence analysis of a subset of oseltamivir-resistant and sensitive virus isolates from both seasons ($n = 725$) showed that 53 (7.3%) had mutations at residue D151 (D → E/G/N), while 9 (1.2%) had mutations at Q136 (Q → K) and 2 (0.3%) had mutations at both residues. Viruses with very high IC_{50} for oseltamivir and peramivir, and elevated IC_{50} for zanamivir, had H274Y in addition to mutations at D151 and/or Q136, residues which can potentially confer NAI resistance based on recent N1 NA crystal structure data. Mutations at D151 without H274Y, did not elevate IC_{50} for any tested NAI, however, Q136K alone significantly reduced susceptibility to zanamivir (36-fold), peramivir (80-fold) and A-315675 (114-fold) but not oseltamivir. Mutations at D151 and Q136 were present only in MDCK grown viruses but not in matching original clinical specimens ($n = 33$) which were available for testing, suggesting that these variants were the result of cell culture selection or they were present in very low proportions. Our findings provide evidence that propagation of influenza virus outside its natural host may lead to selection of virus variants with mutations in the NA that affect sensitivity to NAIs and thus poses implications for drug resistance monitoring and diagnostics.

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1. Introduction

Antiviral drugs occupy an important niche in the management of influenza infections (Moscona, 2008; Abed et al., 2006; Moscona, 2005). They directly target influenza viruses and are effective for treatment when administered early in the course of infection, or for prophylaxis when used soon after exposure (Moscona, 2008). Two classes of antiviral agents are currently licensed for the control of influenza infections: M2 ion channel blockers and neuraminidase inhibitors (NAIs). The M2 blockers (amantadine and rimantadine) are effective against influenza A viruses, but not B viruses (Hayden, 1996). However, the effectiveness of this class of drugs has greatly been compromised by the rapid emergence of resistance among influenza A (H3N2) subtype and among some A (H1N1) viruses circulating in certain geographic areas (Bright et al., 2005; CDC, 2008a; Deyde et al., 2007). So far, all the recently emerged 2009 pandemic H1N1 viruses tested for adamantane resistance at CDC have shown resistance to the drug (CDC, 2009; Dawood et al., 2009; Garten et al., 2009).

Oseltamivir and zanamivir are currently the only drugs approved for use against type A and type B influenza infections (Moscona, 2005; Moscona, 2008), including the novel 2009 pandemic H1N1 viruses (CDC, 2009; Dawood et al., 2009). Oseltamivir is administered orally while zanamivir is inhaled (Smith et al., 2002; Colman, 2005) and therefore not recommended in certain patients including those who are severely ill, young children and the elderly (Freund et al., 1999; Medeiros et al., 2007; Hedrick et al., 2000; Diggory et al., 2001). The availability of an intravenous formulation of zanamivir could be beneficial for such patients, especially in

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instances of oseltamivir resistance (Moscona, 2008). The investigational NAI peramivir, though not yet licensed (Sidwell and Smee, 2002; Babu et al., 2000), is currently undergoing clinical trials utilizing an intravenous or intramuscular formulation.

Emergence of NAI-resistant viruses has previously been low (Hatakeyama et al., 2007; Ward et al., 2005; Whitley et al., 2001) though studies of virus variants collected from young children following oseltamivir treatment revealed 18% resistance (Kiso et al., 2004). However, during the 2007–2008 influenza season (viruses collected October 01, 2007 to September 30, 2008), the emergence and transmission of H274Y oseltamivir-resistant influenza A (H1N1) viruses was simultaneously detected in several countries globally (Dharan et al., 2009; Hauge et al., 2009; Meijer et al., 2009; Sheu et al., 2008; CDC, 2008a,b; Lackenby et al., 2008a; Besselaar et al., 2008). Similar trends were observed during the 2008–2009 season (viruses collected October 01, 2008 to September 30, 2009), with many countries reporting up to 100% oseltamivir resistance in influenza A (H1N1) viruses http://www.who.int/csr/disease/influenza/h1n1_table/en/index.html and http://www.cdc.gov/flu/weekly/. It is therefore critical to enhance surveillance on NAI susceptibility of influenza viruses circulating in different parts of the world, expecially those carrying the N1 enzyme (pandemic H1N1, avian H5N1 and others), since NAIs are currently the only effective antivirals for treatment and chemoprophylaxis of both seasonal and pandemic influenza infections.

Zanamivir, unlike oseltamivir, has been prescribed less often due to current limitations of its use (CDC, 2008b), therefore emergence of resistance to this drug has been rare. Previously, the only reported zanamivir resistance was in an influenza B virus with R152K mutation in the NA which was isolated from a zanamivir-treated immunocompromised patient (Gubareva et al., 1998), and recently a novel Q136K mutation (Hurt et al., 2009) was detected in the NA of influenza A (H1N1) isolates which conferred resistance to zanamivir and peramivir, but had no effect on oseltamivir susceptibility. In addition, zanamivir-resistant mutants have been generated *in vitro*, including E119G, D, A and R292K in influenza N2 (Gubareva et al., 1996, 1997), E119G and R292K in influenza N9 (McKimm-Breschkin et al., 1998; Blick et al., 1995) and E119G, D in influenza B (Cheam et al., 2004; Barnett et al., 1999; Staschke et al., 1995).

Molecular markers of resistance to NAIs are currently less well established compared to M2 blockers; hence resistance to this newer class of drugs is not well characterized. Presently, detection of NAI-resistant viruses in surveillance laboratories is conducted using either the chemiluminescent or the fluorescent NA inhibition (NI) assay where generated IC_{50} values (drug concentration needed to inhibit 50% of NA enzyme activity) of test viruses are compared with those of sensitive control viruses. Elevated IC_{50} values alone are however, not sufficient criteria for defining NAI resistance and should be combined with detection of known molecular markers of resistance by conventional sequencing (McKimm-Breschkin et al., 2003; Monto et al., 2006; Sheu et al., 2008) or pyrosequencing (Lackenby et al., 2008b; Deyde et al., 2009; Deyde and Gubareva, 2009). The clinical relevance of the resistance detected using the NA inhibition assay has not been fully evaluated; nevertheless, it is essential to monitor changes in the NA and their possible effect on virus susceptibility to existing NAIs.

Assessment of drug susceptibility in NA inhibition assays requires the use of cell grown viruses. In the present study we detected a fraction of seasonal human A (H1N1) viruses collected during 2007–2008 and 2008–2009 influenza seasons (October 01, 2007 to September 30, 2009) that carried mutations in the NA not seen in matching clinical specimens, suggesting that even in the absence of drug pressure, propagation of viruses outside of natural host (in MDCK cells) can lead to selection of NA variants with altered susceptibility to NAIs (Hurt et al., 2009). Mechanisms driving such a selective process are not clearly understood, but the phenomenon poses serious implications for the detection of antiviral resistance.

2. Materials and methods

2.1. Viruses and cells

Influenza virus isolates and matching clinical specimens collected during 2007–2008 and 2008–2009 seasons were submitted to the World Health Organization Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the CDC in Atlanta, GA, USA. Virus isolates were propagated further in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) at CDC. Reference viruses A/Georgia/17/2006 (H1N1) and A/Georgia/20/2006 (H1N1) representative of sensitive and oseltamivir-resistant A(H1N1) viruses, respectively, were also propagated in MDCK cells.

2.2. NA inhibitors

Zanamivir was supplied by GlaxoSmithKline (Uxbridge, UK), while oseltamivir carboxylate, the active compound of the ethyl ester prodrug oseltamivir phosphate, was supplied by Hoffmann-La Roche (Basel, Switzerland). Additional investigative inhibitors were used in the study including peramivir (BioCryst Pharmaceuticals, Birmingham, AL) and A-315675 (Abbott Laboratories, Abbott Park, IL).

2.3. NA inhibition assays

Susceptibility of viruses to NAIs was assessed using chemiluminescent NI assay (Buxton et al., 2000). The chemiluminescent NI assay uses a 1,2-dioxetane derivative of sialic acid as substrate and was performed using the NAStar™ Kit (Applied Biosystems, Foster City, CA) as described in detail (Sheu et al., 2008).

2.4. Statistical analysis

Calculation of 50% inhibitory concentration (IC_{50}) values and curve-fitting were performed by Robosage Version 7.31 software (GlaxoSmithKline, inhouse program), an add-in for MS Excel (Microsoft Corp., Redmond, WA) using the equation $y = V_{max} \times \{1 - [x/(K + x)]\}$ as previously described by Sheu et al., 2008; McKimm-Breschkin et al., 2003, where V_{max} is the maximum rate of metabolism, x is the inhibitor concentration, y is the response being inhibited and K is the IC_{50} for the inhibition curve (that is, $y = 50\%\ V_{max}$ when $x = K$).

2.5. Pyrosequencing

Viral RNAs were extracted from 100 μl of viral cell culture supernatant using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and RT-PCR performed with Qiagen™ One-Step RT-PCR Kit (Qiagen, Valencia, CA). Pyrosequencing to detect molecular markers of resistance at codons 136, 151 and 274 in NA were performed on the PSQ96MA platform (Biotage AB, Uppsala, Sweden) as previously described (Deyde et al., 2009). Pyrosequence data consisting of 45–60 nucleotide reads were quantified and background corrected using PSQ96MA version 2.0.2 software (Biotage AB, Uppsala, Sweden). Sequences were aligned and analyzed using DNAStar analysis programs (DNAStar, Madison, WI, USA).

Table 1
Range in IC_{50} for NAIs among oseltamivir-resistant and oseltamivir-sensitive influenza A (H1N1) viruses collected during 2007–2008 and 2008–2009 seasons.

Season	Isolates tested	Range in IC_{50} (nM)[a]			
		Oseltamivir	Zanamivir	Peramivir	A-315675
2007–2008[b]	H274Y mutants	47.65–1431.39 (n = 359)	0.14–10.60 (n = 359)	0.52–591.08 (n = 116)	0.37–34.91 (n = 116)
	H274 wildtype	0.05–2.95 (n = 1494)	0.06–39.86 (n = 1494)	0.06–20.26 (n = 30)	0.13–58.64 (n = 30)
2008–2009[c]	H274Y mutants	34.69–1617.04 (n = 1314)	0.07–18.91 (n = 1314)	0.51–510.57 (n = 134)	0.23 - 16.96 (n = 134)
	H274 wildtype	0.10–1.10 (n = 98)	0.13–0.95 (n = 98)	0.05–0.15 (n = 5)	0.08–0.32 (n = 5)

[a] IC_{50} data generated in chemiluminescent NI assay.
[b] For 2007–2008 season, number of isolates tested for oseltamivir and zanamivir susceptibility (n = 1853) included 359 oseltamivir-resistant and 1494 oseltamivir-sensitive viruses. Number of isolates tested for peramivir and A315675 susceptibility included 116 oseltamivir-resistant and 30 oseltamivir-sensitive viruses, respectively.
[c] For 2008–2009 season (as of September 2009), the number of isolates tested for oseltamivir and zanamivir susceptibility (n = 1412) included 1314 oseltamivir-resistant and 98 oseltamivir-sensitive viruses. Number of isolates tested for peramivir and A315675 susceptibility included 134 oseltamivir-resistant and 5 oseltamivir-sensitive viruses, respectively.

2.6. Sequencing by dideoxy chain termination method

Viral RNAs were extracted from 100 μl of viral cell culture supernatant using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA) and RT-PCR performed with Qiagen™ One-Step RT-PCR Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's directions. Amplified PCR products were purified using ExoSAP-IT® reagent (USB, Cleveland, OH). Sequence template of the PCR product was synthesized using ABI Prism® BigDye™ Terminator Kit (Applied Biosystems, Foster City, CA) and sequencing reactions carried out on the DNA Tetrad2 Engine Thermocycler (Biorad, Hercules, CA). Purification of sequencing product was performed using Centri-Sep 96-well Spin Columns (Princeton Separations, Adelphia, NJ) and sequences were generated in an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Analysis of the electronic sequence data was performed using Lasergene® DNAStar software version 7.0 (DNAStar, Madison, WI, USA).

3. Results

3.1. Susceptibility of seasonal influenza A (H1N1) virus isolates to neuraminidase inhibitors

Virus isolates submitted to the CDC during influenza seasons 2007–2008 (viruses collected October 01, 2007 to September 30, 2008) and 2008–2009 (viruses collected October 01, 2008 to September 30, 2009) were additionally passaged once to twice in MDCK cells and routinely tested for oseltamivir and zanamivir sensitivity in the chemiluminescent NI assay. Isolates that exhibited elevated IC_{50} for oseltamivir and/or zanamivir were retested for both drugs in addition to two investigative NAIs, peramivir and A-315675.

Among 1853 influenza A (H1N1) isolates collected during the 2007–2008 season, 359 (~20%) exhibited elevated IC_{50} for oseltamivir ranging from 47.65 nM to 1431.39 nM (Table 1) which corresponded to approximately 100–3000-fold increase in IC_{50} for a sensitive control virus, A/Georgia/17/2006 (H1N1). Among season 2007–2008 viruses with elevated IC_{50} for oseltamivir, IC_{50} for zanamivir ranged from 0.14 nM to 10.6 nM (up to 20-fold difference compared to the sensitive control virus). Viruses with normal IC_{50} for oseltamivir (0.05–2.95 nM), had zanamivir IC_{50} ranging from 0.06 nM to 39.86 nM.

As of September 2009, 1319 (90%) of 1418 influenza A (H1N1) viruses collected during the 2008–2009 season and tested in the chemiluminescent NI assay were resistant to oseltamivir (IC_{50} 34.69–1617.04 nM). These oseltamivir-resistant viruses exhibited IC_{50} for zanamivir ranged from 0.07 nM to 18.91 nM (up to 40-fold difference compared to the sensitive control virus). Viruses with normal IC_{50} for oseltamivir (0.10–1.10 nM), had zanamivir IC_{50} ranging from 0.13 nM to 0.95 nM.

Pyrosequencing analysis targeted at position 274 in NA revealed the presence of the oseltamivir resistance marker, H274Y (H275Y in N1 numbering) in all the influenza A (H1N1) viruses that exhibited elevated IC_{50} for oseltamivir.

Table 2
Frequency of mutations at positions D151 and Q136 in the neuraminidase of influenza A(H1N1) isolates from seasons 2007 to 2008 and 2008 to 2009.

Influenza season (Sequenced isolates)	Detected variant[a]	No. of detected variants[b]	Mixes among detected variants[c]	Variants with H274Y mutation[d]
2007–2008 (n = 379)	D151E	4 (1.1%)	1 (25.0%)	1 (0.3%)
	D151G	5 (1.3%)	5 (100%)	3 (0.8%)
	D151N	18 (4.7%)	17 (94.4%)	6 (1.6%)
	Q136K	9 (2.4%)	7 (77.8%)	4 (1.1%)
2008–2009 (n = 346)	D151E	3 (0.9%)	3 (100%)	2 (0.6%)
	D151G	5 (1.4%)	5 (100%)	5 (1.4%)
	D151N	18 (5.2%)	18 (100%)	10 (2.9%)
	Q136K	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total[e] (n = 725)	D151E	7 (1.0%)	4 (57.1%)	3 (0.4%)
	D151G	10 (1.4%)	10 (100%)	8 (1.1%)
	D151N	36 (5.0%)	35 (97.2%)	16 (2.2%)
	Q136K	9 (1.2%)	7 (77.8%)	4 (0.4%)

[a] Mutation detected in neuraminidase (NA) of sequenced isolates. The variants with dominant mutation as well as those that are mixes are classified together.
[b] Number of Q136 or D151 variants detected among seasonal A(H1N1) virus isolates with fully sequenced NA. For 2007–2008 season, 283 oseltamivir-sensitive and 96 oseltamivir-resistant viruses were sequenced (n = 379). For 2008–2009 season, 51 oseltamivir-sensitive and 295 oseltamivir-resistant viruses were sequenced (n = 346). Percentage denotes proportion of detected variants among sequenced viruses in each respective season and in total.
[c] Number of Q136 or D151 variants that exhibited both wildtype and mutant nucleotides (mixes) at the same position of the NA sequence in cell grown isolates. Percentage denotes proportion of viruses with mixes among respective variants.
[d] Number of Q136 or D151 variants with additional oseltamivir resistance conferring mutation H274Y. Percentage denotes proportion of variants with additional H274Y mutation among sequenced viruses.
[e] Total; sum of 2007–2008 and 2008–2009 seasons.

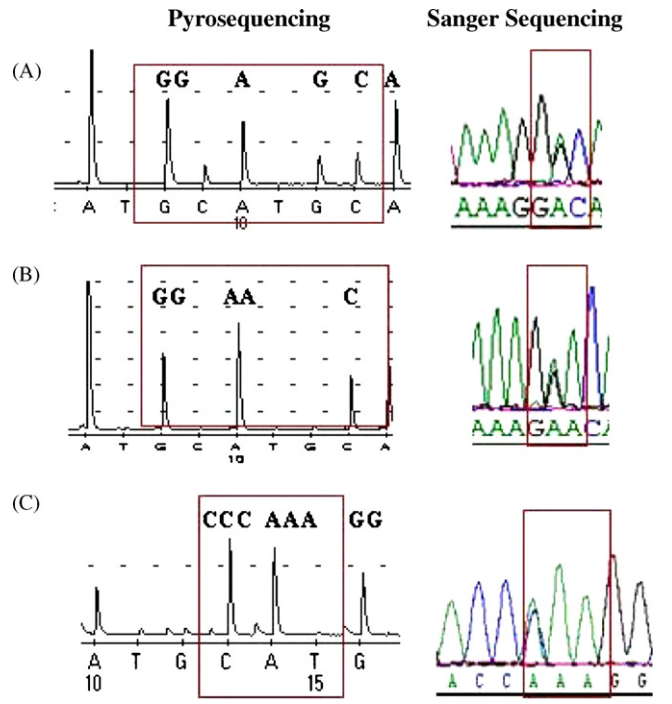

Fig. 1. Pyrograms and chromatograms of NA sequence data showing mixes of mutant and wildtype nucleotide sequences in selected MDCK cell culture grown isolates of (A) A/Montserrat/1448/2008 (H1N1) with a mix of GAC (aspartic acid) and GGC (Glycine), indicating the presence of D151D/G mutation; (B) A/Montserrat/1447/2008 with a mix of GAC (aspartic acid) and AAC (asparagine), indicating the presence of D151D/N; and (C) A/Hong Kong/349/2008(H1N1) with a mix of CAA (glutamine) and AAA (lysine), indicating the presence of Q136Q/K mutation.

3.2. Frequency of D151 and Q136 variants among sequenced influenza A (H1N1) isolates

Full NA sequences were analyzed for a subset of oseltamivir-resistant ($n = 95$) and oseltamivir-sensitive ($n = 281$) viruses from season 2007 to 2008, as well a subset of oseltamivir-resistant ($n = 296$) and oseltamivir-sensitive ($n = 53$) viruses from season 2008 to 2009. As expected, H274Y mutation was present in all viruses with elevated IC_{50} for oseltamivir, while additional mutations were detected at residues D151 and Q136 in some oseltamivir-sensitive and resistant isolates (Table 2).

In the 2007–2008 season, mutations at D151 were more frequent than those observed at Q136, with a total of 27 out of 379 sequenced viruses (7.2%) exhibiting D → E/G/N mutations compared to nine viruses (2.4%) exhibiting Q136K mutation. Similar results were observed in season 2008–2009 where 26 of the 346 sequenced viruses (7.4%) had mutation at D151, however, none of the sequenced viruses from this season exhibited mutation at Q136 (Table 2).

In total, out of the 725 isolates sequenced from both seasons, 53 (7.3%) isolates had mutation at D151 (D → E/G/N), while 9 (1.2%) were Q136K variants (Table 2). However, two viruses, A/Hong Kong/17/2008 and A/Kenya/9309/2008, exhibited mutations at both residues, bringing the actual total of detected variants to 60. Among the 53 D151 variants, 36 (67.9%) were D151N, while 10 (18.9%) were D151G and 7 (13.2%) were D151E.

We observed that some D151E/G/N and Q136K variants were mixes, exhibiting both wildtype and mutant nucleotides at the NA sequence position (Table 2, Fig. 1). Among 2007–2008 viruses, 25% of the detected D151E variants were mixes, as were 100% and 94% of D151G and D151N variants, respectively, while seven of the nine Q136K variants (78%) were mixes. Among season 2008–2009 viruses, all (100%) of the detected D151 variants were mixes.

3.3. Effects of mutations at neuraminidase residues D151 and Q136 on NAI susceptibility

Among 725 sequenced virus isolates from seasons 2007 to 2008 and 2008 to 2009, 27 (3.7%) of the D151E/G/N variants had an additional H274Y mutation, as did four (0.4%) of the Q136K variants (Table 2). Analysis of IC_{50} values generated in the chemiluminescent NI assay showed that such variants exhibited IC_{50} for oseltamivir and peramivir that were significantly higher than those of viruses with only H274Y, the molecular marker for oseltamivir resistance (Tables 3 and 4).

On average, isolates that carried D151G and H274Y mutations exhibited the highest mean IC_{50} for oseltamivir (618.31 nM) which was ~1200-fold and 3-fold higher than those of the sensitive and resistant (H274Y) control viruses, respectively (Table 3). These isolates also exhibited very high average IC_{50} for peramivir (~1000-fold and 9-fold higher than those of sensitive and resistant H274Y control viruses) and reduced susceptibility to zanamivir and A-315675. Isolates with D151N and H274Y exhibited IC_{50}s for oseltamivir and peramivir that were ~800-fold and 2–5-folds higher that those of the sensitive and resistant H274Y control viruses, respectively (Table 3). These isolates were sensitive to zanamivir but showed reduced susceptibility to A-315675. Isolates with D151E and H274Y exhibited IC_{50} for oseltamivir, zanamivir and peramivir that were similar to those of the resistant H274Y control, except for the IC_{50} for A-315675 which was 4-fold more. The combination of Q136K and H274Y mutations greatly reduced peramivir susceptibility, with IC_{50} of 306.87 nM (~1800-fold and 13-fold higher than those of sensitive and resistant H274Y control viruses), while Q136K and H274Y with an additional D151N mutation produced even higher IC_{50} for peramivir (390.94 nM). Both variant combinations had similar impact as H274Y alone, on oseltamivir susceptibility, but resulted in reduced susceptibility to zanamivir and A-315675.

Table 4 shows chemiluminescent NI assay IC_{50} data for selected isolates with mutations at D151 and Q136. The IC_{50} data for all analyzed isolates is provided in the Supplementary material. Among analyzed isolates, A/Thailand/1035/2008 (H1N1) with D151D/G mix in addition to H274Y had the highest IC_{50} for oseltamivir (1617.04 nM), which was ~3000 and ~10 times higher than those of the sensitive and resistant H274Y control viruses, respectively. Isolates A/Monserrat/1447/2008 (H1N1) and A/Monserrat/1448/2008 (H1N1) with H274Y in addition to D151D/G and D151D/N, respectively, exhibited the next highest IC_{50}s for oseltamivir which were ~1900- and ~2800-fold more than that of the sensitive control virus, respectively, and 5–8 times higher than that of the resistant H274Y control virus. The above three isolates also exhibited very high IC_{50} for peramivir (~1700–2000- and 12–15-fold compared to the sensitive and resistant H274Y control viruses, respectively).

The IC_{50} for zanamivir in A/Thailand/1035/2008 (H1N1) and A/Monserrat/1447/2008 (H1N1) with the combination D151G-H274Y were 37- and 12-fold higher than that of the sensitive control virus, respectively, while that of A/Monserrat/1448/2008 (H1N1) with D151N-H274Y was 7-fold higher. All three isolates exhibited reduced susceptibility to A-315675, with 10–70-fold higher IC_{50} than the sensitive control virus.

Isolates with similar mutations, A/Hawaii/20/2008 (H1N1) with H274Y and D151D/G, as well as A/Uruguay/03/2008 (H1N1) with H274Y and D151D/N also exhibited significantly high IC_{50}s for oseltamivir and peramivir, and reduced susceptibility to zanamivir and A-315675 (Table 4). However, the isolate A/Hawaii/28/2007 with D151E and H274Y mutations exhibited IC_{50} for oseltamivir, peramivir and zanamivir that were similar to those of the

Table 3
Effects of detected neuraminidase mutations on NAI susceptibility.

Mutation[a]	Chemiluminescent NI assay Mean IC_{50}, nM (fold difference[c])			
	Oseltamivir	Zanamivir	Peramivir	A-315675
D151E ($n=3$)	0.30 (1)	0.35 (1)	0.07 (0)	0.99 (2)
D151G ($n=2$)	0.41 (1)	0.83 (2)	0.05 (0)	0.13 (0)
D151N ($n=20$)	0.50 (1)	0.54 (1)	0.07 (0)	0.16 (0)
D151E + H274Y ($n=3$)	120.05 (231)	0.43 (1)	13.54 (80)	5.85 (13)
D151E + Q136K ($n=1$)	0.15 (0)	12.60 (25)	3.10 (18)	12.02 (27)
D151G + H274Y ($n=8$)	618.31 (1189)	6.92 (14)	197.30 (1161)	8.32 (18)
D151N + H274Y ($n=15$)	415.47 (799)	1.28 (3)	122.08 (718)	3.50 (8)
Q136K ($n=4$)	0.20 (0)	18.43 (36)	13.53 (80)	51.49 (114)
Q136K + H274Y ($n=2$)[b]	102.74 (198)	7.56 (15)	306.87 (1805)	33.44 (74)
D151N + H274Y + Q136K ($n=1$)	184.92 (356)	2.02 (4)	390.94 (2300)	4.99 (11)
A/Georgia/20/2006, resistant control	189.33 (364)	0.95 (2)	22.61 (133)	1.45 (3)
A/Georgia/17/2006, sensitive control	0.52 (1)	0.51 (1)	0.17 (1)	0.45 (1)

[a] Mutation or combination of NA mutations detected in sequenced isolates. The variants with dominant mutations as well as those that are mixes have been classified together.
[b] A single isolate, A/Montana/01/2008 which exhibited mixes Q136Q/K and H274H/Y, had an uncharacteristically low IC_{50} for oseltamivir (14.97 nM) and was excluded from the statistical analysis for IC_{50} data.
[c] Compared to IC_{50} values determined for sensitive control A/Georgia/17/2006 (H1N1) (Sheu et al., 2008).

oseltamivir-resistant control virus with only H274Y, with the exception of A-315675 whose IC_{50} was 8-fold greater.

Among analyzed isolates, the highest IC_{50} for peramivir (478.43 nM) was observed in the isolate A/Hong Kong/349/2008 (H1N1) which harbored H274Y and Q136Q/K. This was ~3000- and 20-fold more compared to the sensitive and resistant H274Y control viruses, respectively. However, this isolate exhibited IC_{50} for oseltamivir which was not significantly different from that of the resistant H274Y control, and IC_{50} for zanamivir and A-315675 that were 10- and 26-fold higher, respectively. Another isolate, A/Hong Kong/17/2008 (H1N1), with H274Y in addition D151D/N and Q136Q/K exhibited similar results for peramivir and oseltamivir, but only 2–4-fold difference in IC_{50} for zanamivir and A-315675 compared to the that of the resistant H274Y control.

In the absence of H274Y, however, D151E/G/N mutations had no apparent effect on any of the four NAIs, as IC_{50} values were not enhanced in such isolates (Tables 3 and 4). Among these variants, no significant difference was observed between mutations that were dominant (mutant residues only) or mixed (wildtype plus mutant residues).

In contrast, Q136K by itself reduced zanamivir susceptibility the most (Tables 3 and 4). The lone Q136K mutation in A/Panama/1310/2008 (H1N1) (Table 4), resulted in substantial reduction in susceptibility to zanamivir, peramivir and A-315675 (78-, 119- and 130-fold, respectively, compared to the sensitive control), but had no effect on sensitivity to oseltamivir (IC_{50} = 0.16 nM). Similarly, A/Kenya/9309/2007 (H1N1) with mixes Q136Q/K and D151D/E, in absence of H274Y, exhibited moderate

Table 4
Effects of mutations at D151 and Q136 in the neuraminidase on drug susceptibility of seasonal influenza A (H1N1) viruses.

Strain designation	Accession*	Passage[a]	Mutations at Q136	Mutations at D151	Other NA mutations	Chemiluminescent NI assay IC_{50}, nM (fold difference[b])			
						Oseltamivir	Zanamivir	Peramivir	A-315675
A/Panama/1310/2008	GQ423405	C2/C1	Q136K	–	–	0.16 (0)	39.86 (78)	20.26 (119)	58.64 (130)
A/Hong Kong/349/2008	GQ423401	C2/C1	Q136Q/K	–	H274Y	105.93 (204)	10.31 (20)	478.43 (2814)	34.91 (78)
A/Kenya/9309/2007	GQ423402	C2/C1	Q136Q/K	D151D/E		0.15 (0)	12.60 (25)	3.10 (18)	12.02 (27)
A/Hong Kong/17/2008	GQ423395	C2/C1	Q136Q/K	D151D/N	H274Y	184.92 (356)	2.02 (4)	390.94 (2300)	4.99 (11)
A/Hawaii/28/2007	EU516125	C1/C1	–	D151E	H274Y	129.32 (249)	0.47 (1)	24.53 (144)	10.82 (24)
A/Thailand/1035/2008	GQ423407	C2/C1	–	D151D/G	H274Y	1617.04 (3110)	18.91 (37)	323.11 (1901)	4.44 (10)
A/Montserrat/1447/2008	GQ423399	X/C1	–	D151D/G	H274Y	991.73 (1907)	6.28 (12)	280.48 (1650)	31.64 (70)
A/Hawaii/20/2008	GQ423418	C2/C1	–	D151D/G	H274Y	524.62 (1009)	16.10 (32)	321.52 (1891)	5.77 (13)
A/Montserrat/1448/2008	GQ423400	X/C2	–	D151D/N	H274Y	1431.39 (2753)	3.35 (7)	335.63 (1974)	11.62 (26)
A/Uruguay/03/2008	GQ423404	C2/C1	–	D151D/N	H274Y	393.07 (756)	393.07 (756)	74.94 (441)	2.02 (4)
A/Hawaii/19/2008	FJ549057	C2/C1	–	D151D/N	H274Y	460.09 (885)	0.99 (2)	107.06 (630)	3.22 (7)
A/Iowa/01/2009	GQ423406	C1/C1	–	D151D/N	H274Y	364.13 (700)	1.19 (2)	133.49 (785)	3.93 (9)
A/Hong Kong/179/2008	GQ423412	C2/C1	–	D151E	–	0.32 (1)	0.29 (1)	0.11 (1)	2.84 (6)
A/Hong Kong/84/2009	GQ423408	C2/C1	–	D151D/E	–	0.33 (1)	0.48 (1)	0.11 (1)	0.14 (0)
A/Taiwan/401/2008	GQ423403	C3/C1	–	D151D/G	–	0.35 (1)	0.96 (2)	0.1 (1)	0.26 (1)
A/Japan/763/2008	GQ423398	X/C2	–	D151N	–	1.28 (2)	0.95 (2)	0.23 (1)	0.53 (1)
A/Hong Kong/36/2008	GQ423396	C2/C1	–	D151D/N	–	1.29 (2)	1.29 (2)	0.23 (1)	0.65 (1)
A/Georgia/20/2006, resistant control	EU516199	–	–	–	H274Y	189.33 (364)	0.95 (2)	22.61 (133)	1.45 (3)
A/Georgia/17/2006, sensitive control	EU100630	–	–	–	Wildtype	0.52 (1)	0.51 (1)	0.17 (1)	0.45 (1)

* GenBank.
[a] Isolate passage: C1/C1 – Isolate passaged once at submitting laboratory, then once in MDCK cells at CDC.
C2/C1 – Isolate passaged twice at submitting, then once in MDCK cells at CDC.
M1/C1 - Isolate grown in MDCK cells at submitting lab, then passaged once in MDCK cells at CDC.
X/C1 - Isolate grown in cells at submitting lab (cell type, passage number unknown), then passaged once in MDCK cells at CDC.
X/C2 - Isolate grown in cells at originating laboratory (cell type, passage number unknown), then passaged twice in MDCK cells at CDC.
[b] Compared to IC_{50} values determined for sensitive control A/Georgia/17/2006 (H1N1) (Sheu et al., 2008).

Table 5
Neuraminidase nucleotide sequences of virus isolates and matching original clinical specimens.

Variant[a]	No. of variants among sequenced isolates[b]	Sequence of variant	No. of matching clinical specimens[c]	Sequence of clinical specimen
D151E	7	GAA	3	GAC
D151G	10	GGC	4	GAC
D151N	36	AAC	21	GAC
Q136K	9	AAA	5	CAA

[a] Mutation detected in sequenced isolates. The variants with dominant mutation as well as those that are mixes are classified together.
[b] Total number of variants detected out of 725 sequenced virus isolates from 2007 to 2008 and 2008 to 2009 seasons.
[c] Number of matching original clinical specimens that were available for testing.

fold increases in IC_{50} for zanamivir, peramivir and A-315675 (25-, 18- and 27-fold, respectively, when compared to a sensitive control virus), but was sensitive to oseltamivir (IC_{50} = 0.15 nM).

3.4. NA sequence analysis of matching clinical specimens

To investigate in detail the emergence of D151 and Q136 variants, original clinical specimens matching virus isolates in which variants were detected, were analyzed by pyrosequencing and conventional sequence analysis. Results of both analyses showed that mutations at residues Q136 and D151 were present only in the MDCK grown isolates (n = 60), but not in the 33 matching original clinical specimens that were available for testing (Table 5). However, the mutation at residue H274 was present in both cell culture grown isolates and original clinical specimens (data not shown).

4. Discussion

Propagation of influenza viruses outside of their natural host has been shown to provide a growth advantage to variants with mutations in the HA, a receptor-binding viral protein (Deom et al., 1986; Gubareva et al., 1994, 2001; Katz et al., 1987; Kodihalli et al., 1995; Schild et al., 1983; Meyer et al., 1993; Robertson et al., 1994). In the present study, we provide evidence that propagation of seasonal human A(H1N1) viruses in MDCK cells can lead to the emergence of virus variants carrying substitutions in the receptor-destroying protein, NA.

Presently, monitoring susceptibility to the newer anti-influenza drugs, oseltamivir and zanamivir, is conducted in cell culture propagated viruses and is based on IC_{50} values determined in the NA inhibition assay. Although this assay does not accurately reproduce physiological conditions and utilizes a small synthetic substrate (NAStar™ or MUNANA) (Bronstein et al., 1991; Buxton et al., 2000; Potier et al., 1979), the attained IC_{50} values are often used to assess the degree of resistance to a particular drug and to predict clinical relevance of such resistance (Hurt et al., 2007; Monto et al., 2006; Mungall et al., 2003, 2004; Sheu et al., 2008; Wetherall et al., 2003).

In this study, propagation of seasonal influenza A(H1N1) viruses outside their natural host (human) prior to testing in the chemiluminescent NI assay led to the emergence of cell culture selected virus variants, some of which exhibited reduced susceptibility to NAIs. We detected the emergence of viruses with Q136K and/or D151E/N/G mutations in the NA which were evident only in cell culture propagated virus isolates, but not in matching original clinical specimens. The NA residues Q136 and D151 reside close to or within the 150-loop of the NA enzyme active site, respectively, and based on crystal structure data, these residues were recently identified as vulnerable to NAI resistance emergence in the enzyme of N1 subtype (Luo, 2006; Russell et al., 2006).

The mutation D151G together with H274Y, dramatically enhanced resistance to oseltamivir and peramivir, and slightly reduced susceptibility to zanamivir and A-315675, while mutation D151N combined with H274Y also enhanced resistance to oseltamivir and peramivir, but not zanamivir or A-315675. However, D151E in combination with H274Y only slightly enhanced resistance to A-315675, but had no apparent effect on the other three tested NAIs (Table 4). D151G and D151N, but not D151E potentiated the effects of H274Y on NAI susceptibility and conferred cross-resistance to four NAIs. However, substitutions at D151 when present alone did not affect susceptibility to either of four NAIs used in the study, but in combination with the H274Y mutation, they substantially enhanced resistance to oseltamivir and peramivir. Elevated IC_{50} values in viruses containing mutations at D151 have been reported in previous surveillance studies (McKimm-Breschkin et al., 2003; Sheu et al., 2008), but questions regarding the role of such substitutions in NAI resistance still remain.

The mutation Q136K in combination with H274Y, or by itself, conferred cross-resistance to zanamivir, peramivir and A-315675, but had no effect on inhibition by oseltamivir (Table 3). The cell culture selected variant A/Panama/1310/2008 (H1N1) with the Q136K mutation in absence of H274Y exhibited reduced susceptibility to zanamivir, peramivir and A-315675 but not to oseltamivir (Table 4). This result is in accordance with a previous study (Hurt et al., 2009) where Q136K mutation showed no effect on oseltamivir susceptibility but caused approximately a 300-fold and a 70-fold reduction in zanamivir and peramivir susceptibility, respectively, in the fluorescent NI assay.

By either conventional sequencing (Sheu et al., 2008; McKimm-Breschkin et al., 2003) or pyrosequencing (Lackenby et al., 2008b; Deyde et al., 2009; Deyde and Gubareva, 2009) which can detect as low as 10% of virus variants carrying a resistance conferring mutation, mutations D151E/G/N and Q136K were only detected in the grown virus isolates but not the matching original clinical specimens, suggesting that <10% of such variants were present in the unpropagated clinical specimens. Our results concur with previous observations (Gubareva et al., 1998, 2001; Hurt et al., 2009) where the emergence of the NAI-resistant mutants was more readily detectable in the MDCK-propagated virus but not in the matching clinical specimens. In addition, D151E mutation was detected in the NA of cell grown but not the matching clinical specimen of a seasonal influenza A (H1N1) virus, A/Hawaii/28/2007 (Deyde et al., 2009). Depending on the time point when the clinical specimen was collected, drug-resistant variants could be present as clearly dominant or minor populations in original clinical specimens and/or virus isolates in cell culture. (Deyde et al., 2009; Gubareva et al., 1994, 1998; Kiso et al., 2004; Lackenby et al., 2008b).

The majority of D151 and Q136 variants detected in this study were mixes (Table 2), exhibiting almost equal proportions of both wildtype and mutant nucleotides on the NA sequence position (Fig. 1). Even though mutations existing as codominant mixes did not appear to affect NAI IC_{50} values any differently from dominant mutations (Table 4), mixes may affect the accuracy of setting definitive criteria for diagnosis of antiviral resistance. Such mixed mutations can evolve to dominant mutant genotypes with further passage of virus isolates, however, that was not the scope of this study. The majority of viruses we analyzed originated from diverse regions, with some isolates having uncertain passage histories. Mechanisms underlying the preferential growth of some

NA-mutants in MDCK cells (Gubareva et al., 1994, 2001; Hurt et al., 2009) are not well understood but the phenomenon needs to be taken into consideration to ensure accurate diagnosis and reporting of antiviral resistance. Our results highlight the potential consequences of cell culture propagation of viruses prior to NI testing and the effects that such an approach can have on diagnosis of NAI resistance, especially over-estimation of resistance.

Viruses with reduced NAI susceptibility can emerge as a result of drug use and/or natural sequence variation in the NA. Even in the absence of drug pressure, propagation of virus outside of natural host (i.e., MDCK cells) can lead to selection of NA variants with altered susceptibility to NAIs (Hurt et al., 2009). In this study, limited information available for a few cases indicated that patients were not treated with antivirals prior to collection of the specimens; therefore the NA variants emerged as a result of virus propagation in cell culture. However, we cannot rule out that such mutants can also emerge following treatment with NAIs. In N1 subtype especially, natural variation in the absence of any drug selective pressure may result in less pronounced but significant variations in the sensitivity of influenza viruses to anti-NA drugs (Le et al., 2008; Rameix-Welti et al., 2006). An example are the oseltamivir-resistant H274Y viruses, whose genesis remain unknown, but have previously been isolated from drug-treated patients (Gubareva et al., 2001; Ward et al., 2005; Weinstock et al., 2003; Whitley et al., 2001) and more recently from untreated patients (Sheu et al., 2008; Dharan et al., 2009; Lackenby et al., 2008a; Besselaar et al., 2008). Consequently, in addition to monitoring of the emergence of drug-resistant viruses in patients under treatment, systematic evaluation of the impact of natural genetic variation on the sensitivity to anti-NA drugs should be performed, especially for rapidly evolving, potentially pandemic influenza viruses.

These findings emphasize the importance of collection and preservation of clinical specimens to facilitate efficient and reliable diagnosis of resistance to this newer class of anti-influenza drugs (NAIs). However, clinical specimens matching virus isolates are not always available for testing. In this study, only 33 clinical specimens were available that matched the 60 isolates in which mutations at D151 and Q136 were detected. When only a virus isolate is available for testing, it would be reported to have reduced susceptibility to NAIs, without knowledge of whether the mutation also exists in the matching clinical specimen. Therefore, our study emphasizes a need for caution in reaching conclusion based on incomplete information. With current ongoing clinical trials in South East Asia and other countries on the use of NAIs in A(H5N1) influenza infections, the lessons learned herein can also be applied in the definition of NAI resistance in avian influenza which also contains NA of N1 subtype, and in the 2009 pandemic influenza H1N1 viruses.

Our results highlight the challenges of finding markers of NAI resistance (established and/or novel) in the virus isolate but not in the matching clinical specimen and how that may affect the accuracy of resistance diagnosis. These findings emphasize the necessity to detect NAI resistance conferring mutations in original clinical virus specimens prior to their propagation in cell culture, through sensitive molecular techniques such as pyrosequencing, in order to avoid over-estimation of resistance. However, incomplete knowledge of molecular markers of resistance and type/subtype and even strain specific nature of resistance to NAIs (McKimm-Breschkin, 2005; Jackson et al., 2005; Yen et al., 2007) restricts the usefulness of sequence-based assays; hence the clinical relevance of some mutations identified in the NA remains uncertain. Since the sensitivity of the current phenotypic assay does not allow drug resistance testing directly in clinical specimens, there is a need for cell culture systems that do not promote the selection of virus variants and adequately reflect the susceptibility status of the virus in the human host. Nevertheless, there is a need to define harmonized criteria for diagnosis of resistance through sharing resistant reference strains and utilization of similar methods for assessing NAI susceptibility.

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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.2009.11.005.

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