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Cell Culture-Selected Substitutions in Influenza A(H3N2) Neuraminidase Affect Drug Susceptibility Assessment

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Assessment of drug susceptibility has become an integral part of influenza virus surveillance. In this study, we describe the drug resistance profile of influenza A(H3N2) virus, A/Mississippi/05/2011, collected from a patient treated with oseltamivir and detected via surveillance. An MDCK cell-grown isolate of this virus exhibited highly reduced inhibition by the neuraminidase (NA) inhibitors (NAIs) oseltamivir (8,005-fold), zanamivir (813-fold), peramivir (116-fold), and laninamivir (257-fold) in the NA inhibition assay. Sequence analysis of its NA gene revealed a known oseltamivir-resistance marker, the glutamic acid-to-valine substitution at position 119 (E119V), and an additional change, threonine to isoleucine at position 148 (T148I). Unlike E119V, T148I was not detected in the clinical sample but acquired during viral propagation in MDCK cells. Using recombinant proteins, T148I by itself was shown to cause only a 6-fold increase in the zanamivir 50% inhibitory concentration (IC_{50}) and had no effect on inhibition by other drugs. The T148I substitution reduced NA activity by 50%, most likely by affecting the positioning of the 150 loop at the NA catalytic site. Using pyrosequencing, changes at T148 were detected in 35 (23%) of 150 MDCK cell-grown A(H3N2) viruses tested, which was lower than the frequency of changes at D151 (85%), an NA residue previously implicated in cell selection. We demonstrate that culturing of the A(H3N2) viruses ($n = 11$) at a low multiplicity of infection delayed the emergence of the NA variants with changes at position 148 and/or 151, especially when conducted in MDCK-SIAT1 cells. Our findings highlight the current challenges in monitoring susceptibility of influenza A(H3N2) viruses to the NAI class of antiviral drugs.

Neuraminidase (NA) inhibitors are the only recommended antiviral medications for the control of influenza virus infections due to a high level of resistance to the M2 blocker class of antivirals (1–3). Monitoring susceptibility to NA inhibitors is a critical component of influenza surveillance activities conducted globally. At present, zanamivir and oseltamivir are the only NA inhibitors approved in the United States to control influenza A and B virus infections. Two additional NA inhibitors, laninamivir and peramivir, have been approved for use in Japan, and peramivir is also marketed in South Korea and China.

Susceptibility to NA inhibitors is currently assessed by the NA inhibition (NI) assay, which requires viruses to first be propagated in cell culture (4). Many cell lines have been used for the propagation of influenza viruses, including baby hamster kidney (BHK-21) cells, rhesus monkey kidney epithelial (LLC-MK2) cells, and African green monkey kidney (Vero) cells (5–8). The use of human colon intestinal epithelial (CaCo-2) cells for isolation and propagation of seasonal A(H3N2) viruses has also been advocated in recent years (9, 10). Madin-Darby canine kidney (MDCK) cells, however, remain the most commonly used cell line for the propagation of influenza viruses due to their superior overall sensitivity, high virus yield, and ease of culture and maintenance (11–14). MDCK cells express receptors with both α2-3- and α2-6 neuraminic acid (NeuAc) linkages, making them suitable for isolation of a wide variety of influenza viruses (15). Indeed, MDCK cells are routinely used for virus propagation by the Centers for Disease Control and Prevention (CDC) Influenza Division as part of its surveillance activities. However, the density of α2-6-linked NeuAc receptors is lower than that of the upper respiratory epithelium in humans (16), and this renders the traditional MDCK cell-based assays unsuitable for assessing the NA inhibitor susceptibility of human influenza viruses (17–19).

To address this problem, MDCK cells have been modified to overexpress α2-6-linked NeuAc receptors (20, 21). Although this modification did not cause MDCK-SIAT1 (SIAT1) cells to be a reliable substrate for NA inhibitor susceptibility testing, the modified cell line was found to be advantageous for virus isolation and propagation in a number of laboratories (22). Importantly, propagation of influenza A(H3N2) viruses in MDCK cells has recently been linked to selection of NA variants carrying substitutions at residue D151 (23, 24). Furthermore, the D151G substitution was shown to concurrently reduce NA catalytic activity and increase NA binding to α2-3-linked receptors (24), switching the NA function from receptor destroying to receptor binding. The emergence of the D151G variant has been shown to interfere with hemagglutinin (HA) antigenic analysis (23), which is a critical component of virus surveillance.

The present study demonstrates the emergence of NA variants carrying substitutions at residue T148 as a consequence of A(H3N2) virus culture in MDCK cells. The effects of the T148I substitution on drug susceptibility assessment and NA catalytic activity were investigated. In addition, A(H3N2) viruses were propagated and subsequently passaged using SIAT1 cells to determine if the emergence of

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NA variants with changes at positions 148 and 151 could be mitigated.

MATERIALS AND METHODS

Viruses and cells. Influenza virus isolates and their respective clinical specimens collected during the 2010–2011 season were submitted to the World Health Organization (WHO) Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the CDC for antigenic and genetic characterization and antiviral testing. MDCK (CCL-34; ATCC, Manassas, VA) and MDCK-SIAT1 (a kind gift from M. Matrosovich) cells were maintained by the Scientific Products and Support Branch at the CDC. Viruses were propagated according to standard procedures as described previously (25) with minor modifications. In brief, MDCK cells seeded in T75 flasks were inoculated with 0.6 ml of a clinical specimen, and following 1 h of virus adsorption, 20 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% bovine serum albumin (BSA), 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin was added to the flask. The cells were observed for cytopathic effect (CPE), and the supernatants were harvested no later than 6 days postinoculation. If the HA titer was insufficient to conduct an HA inhibition assay, an additional passage was performed up to four passages in total.

In this study, a modified procedure was used to isolate viruses from clinical specimens and to perform further passage. MDCK and SIAT1 cell monolayers in T25 flasks were washed twice with phosphate-buffered saline (PBS). The clinical specimen (100 μl) was diluted in 900 μl of PBS and added to the flask. After a 1-h virus adsorption at 37°C, the inoculum was removed, the cells were washed twice with PBS, and 7 ml of supplemented DMEM (see above) was added to the flask. The infected cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 6 days and monitored daily for CPE. When the CPE reached 25 to 50%, the supernatants were harvested and clarified by low-speed centrifugation (see Table S1 in the supplemental material). Aliquots were stored at −80°C before testing. Viral HA titers were determined by a standard procedure using guinea pig red blood cells. Infectious-virus titers were determined by limiting dilution in SIAT1 cells and calculated using the Spearman-Karber method (26). A subsequent passage was carried out at a multiplicity of infection (MOI) equal to 0.001 50% tissue culture infective dose ($TCID_{50}$) per cell in a T25 flask.

The CDC reference A(H3N2) virus A/Texas/12/2007 variant carrying the oseltamivir resistance marker, E119V, in the NA and A/Washington/01/2007 wild-type virus were used as controls in the NI assay.

Fisher's exact tests to compare the proportions of neuraminidase variants that emerged at positions T148 and D151 after subsequent passages using different procedures and cell lines were performed using SAS version 9.3 (SAS Institute, Cary, NC) at a significance level where α was equal to 0.05. *P* values of <0.05 were considered statistically significant.

Susceptibility to NA inhibitors. Propagated virus isolates were tested using the fluorescent NI assay with an NA-Fluor kit (Applied Biosystems, Carlsbad, CA, USA), as described previously (27). Four NA inhibitors, zanamivir (GlaxoSmithKline, Uxbridge, United Kingdom), oseltamivir carboxylate (F. Hoffmann-La Roche Ltd., Basel, Switzerland), peramivir (BioCryst Pharmaceuticals, Durham, NC), and laninamivir (R-125489; Biota, Victoria, Australia), were utilized. The IC_{50} (the drug concentration required to inhibit NA activity by 50%) was determined using JASPR v1.2, CDC-developed curve-fitting software, as described previously (27). Means and standard deviations (SD) were calculated from results collected from at least three independent tests.

Criteria set by the WHO Antiviral Working Group (AVWG) were used for interpretation of IC_{50}s (28). Briefly, for influenza type A viruses, an increase in the IC_{50} compared to a susceptible reference virus of <10-fold corresponds to normal inhibition, an IC_{50} with a 10- to 100-fold increase corresponds to reduced inhibition, and a >100-fold increase is equal to highly reduced inhibition.

Sequence analysis. Conventional sequence analysis of the NA gene for A/Mississippi/05/2011 (clinical specimen, passage 2 [P2], and P3) was performed by the Sequencing Activity Team of the Influenza Division at the CDC, as described previously (29). In addition, the HA and NA gene sequences were generated for a subset of A(H3N2) clinical specimens ($n = 11$) and their respective isolates passaged five times in MDCK cells with the use of a primer set (see Table S2A in the supplemental material). Reverse transcription (RT)-PCR amplicons were treated with ExoSap-It reagent following the manufacturer's recommendations (USB, Cleveland, OH) and used as templates in sequencing reactions with an ABI Prism BigDye Terminator kit (Applied Biosystems, Foster City, CA). The products of the sequencing reactions were treated with XTerminator solution (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Sequences generated in an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA) were assembled into fragment-specific contigs using Lasergene software, version 7.0 (DNAStar, Madison, WI).

Pyrosequencing. Viral-RNA extraction, RT-PCR, and pyrosequencing were performed as previously described (30). Pyrosequencing was performed using the PyroMark PSQ96MA instrument (Qiagen, Valencia, CA). The RT-PCR and sequencing primers for analysis of the residue at position 119 in NA (see Table S2B in the supplemental material) were previously described (30). A set of primers for the analysis of residues 148 and 151 in NA were designed with the use of Pyrosequencing Assay Design software (Qiagen, Valencia, CA). Pyrosequencing with customized nucleotide dispensation order was used to detect variants with changes at codons 148 and 151 (29). The nucleotide dispensation AGTAGCACATA GTCACGATCATCGTCTATCA$(GATC)_5$ enabled identification of the following NA variants: threonine (T; nucleotide sequence ACA), lysine (K; nucleotide sequence AAA), or isoleucine (I; nucleotide sequence ATA) at position 148 and aspartic acid (D; nucleotide sequence GAT), glycine (G; nucleotide sequence GGT), or asparagine (N; nucleotide sequence AAT) at position 151. Single-nucleotide polymorphism (SNP) analysis was done essentially as previously described (29) with the limit of detection set at 10%.

Generation of recombinant NAs. A codon-optimized cDNA encoding the ectodomain (residues 82 to 469) of the NA gene of A/Perth/10/2010 (H3N2) (Life Technologies, NY) was subcloned into the pIEx-4 vector (EMD Millipore, MA) using the In-Fusion HD cloning system (Clontech, CA). All subsequent NA mutants (E119V, T148I, and E119V/T148I) were generated from this wild-type pIEx-4-NA clone using a QuikChange Lightning site-directed mutagenesis kit (Agilent, CA). The resulting constructs were transiently transfected into sf9 cells (EMD Millipore, MA) using the Cellfectin II transfection reagent (Life Technologies, NY) according to the manufacturer's protocols. Five days posttransfection, the supernatant containing secreted recombinant N2 NA (recNA) was harvested and clarified, and the recNA was quantified by Western blot analysis, using baculovirus-produced/purified A/Perth/16/2009 NA protein as a control. The supernatants were assessed for functional activity using the MUNANA assay (27) and analyzed without further purification in neuraminidase inhibition assays.

Nucleotide sequence accession numbers. The sequences of the NA genes for A/Mississippi/05/2011 (clinical specimen, P2, and P3) were deposited at the Global Initiative on Sharing All Influenza Data (GISAID), and the accession numbers are given in Table 1. The HA and NA gene sequences for a subset of A(H3N2) clinical specimens ($n = 11$) were deposited at GISAID, and the accession numbers are given in Table 2 and also in Table S3 in the supplemental material.

RESULTS

Detection of an oseltamivir-resistant A(H3N2) virus. To monitor influenza virus susceptibility to NA inhibitors, MDCK cell-grown viruses are tested in the fluorescent NI assay. Among the influenza A(H3N2) viruses tested ($n = 1{,}777$) during the 2010–2011 season, one virus, A/Mississippi/05/2011 (0.06%), exhibited

TABLE 1 Characterization of the A/Mississippi/05/2011(H3N2) virus passaged on MDCK cells

A(H3N2) virus name	Mean IC_{50} ± SD[a] [nM (fold[b])]				NA residue (%)[c]	
	Zanamivir	Oseltamivir	Peramivir	Laninamivir	119 (E/V)	148 (T/I)
A/Mississippi/05/2011 (P0)[d]	NA[e]	NA	NA	NA	28/72	100/0
A/Mississippi/05/2011 (P2)	1.19 ± 0.01 (5)	65.36 ± 13.07 (726)	0.20 ± 0.01 (2)	0.79 ± 0.08 (**3**)	0/100	35/65
A/Mississippi/05/2011 (P3)	195.00 ± 7.47 (813)	720.45 ± 57.71 (8,005)	10.42 ± 1.34 (116)	71.85 ± 3.63 (257)	0/100	0/100
Reference viruses						
A/Washington/01/2007	0.24 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.28 ± 0.03	100/0	57/43
A/Texas/12/2007 (E119V), oseltamivir resistant	0.40 ± 0.02	45.48 ± 9.70 (505)	0.11 ± 0.01	0.34 ± 0.03	0/100	100/0

[a] Based on at least three independent experiments.
[b] Fold increase based on comparison to the respective IC_{50}s of the reference virus, A/Washington/01/2007.
[c] Proportion of NA variants determined using SNP pyrosequencing analysis.
[d] P0, clinical specimen; P2 and P3, virus isolates passaged two and three times, respectively, on MDCK cells using the standard procedure. The GISAID accession numbers for the NA genes are EPI408578, EPI366355, and EPI408583.
[e] NA, not applicable; neuraminidase inhibition testing was not performed on the clinical specimen.

a 726-fold increase in oseltamivir IC_{50} compared to that of the CDC reference drug-sensitive control, A/Washington/01/2007 A(H3N2) virus (Table 1). Sequence analysis of the NA gene of A/Mississippi/05/2011 (P2) revealed the presence of the E119V substitution, a known marker of oseltamivir resistance in A(H3N2) viruses (4). To enable further studies, A/Mississippi/05/2011 (P2) was passaged an additional time, and the P3 isolate was tested in the NI assay. The P3 isolate not only exhibited a very high oseltamivir IC_{50} (8,005-fold), it also showed >100-fold increases in the IC_{50}s of the remaining three NA inhibitors, zanamivir, peramivir, and laninamivir (Table 1). According to the WHO AVWG criteria (28), this result would be interpreted as "highly reduced" inhibition of all four NA inhibitors tested. Notably, NA sequence analysis of the P3 isolate revealed an additional substitution, T148I (nucleotide change, ACA→ATA). To determine if either of the NA substitutions detected in the P3 isolate was present in the clinical specimen, a pyrosequencing assay was performed. The E119V NA variant was detected in the clinical specimen and the P2 and P3 isolates (72%, 100%, and 100%, respectively). In contrast, no changes at position 148 were detected in the clinical specimen, while T148I was present at 65% and at 100% in the P2 and P3 isolates (Table 1). Taken together these data demonstrate that two events had occurred during virus propagation in MDCK cells, an increase in the proportion of the oseltamivir-resistant E119V NA variant and the emergence of the T148I NA variant.

Frequencies of NA substitutions at T148 and D151 in MDCK cell-grown viruses. To assess the frequency of changes at T148 in cell-cultured viruses, NA sequences for A(H3N2) viruses collected globally in the 2010–2011 influenza season and deposited at the GISAID were analyzed. The NA variants with I, K, P, S, or M at residue 148, either alone or in a mixture with the wild type, were detected in 121 (8.8%) of 1,370 sequences analyzed. Because the conventional sequencing is not sufficiently sensitive at detecting viral subpopulations, the pyrosequencing assay was used for the analysis. To this end, the NA sequences of 150 viruses that had undergone between one and four passages in MDCK cells prior to testing (see Table S4 in the supplemental material) were analyzed by pyrosequencing. The analysis revealed that 35 of the 150 (23%) contained an amino acid substitution at codon T148 (I, K, or P), either alone or in a mixture with the wild type (see Table S4). In addition, an even a greater number of NA sequences (85%) showed variance at residue D151 (see Table S4). It has previously been shown that the substitution D151G alters the properties of the viral NA by reducing catalytic activity while increasing the binding affinity for α2,3-sialosides (24). Therefore, it is not unreasonable to expect that the emergence of NA variants with substitution at T148 may have a similar underlying mechanism. Accordingly, we hypothesized that two approaches are likely to put the unwanted T148I and D151G variants at a selective disadvantage: (i) using a lower MOI and (ii) using MDCK cells modified to overexpress α2,6 receptors (SIAT1 cells).

Comparative analysis of MDCK cell- and SIAT1 cell-grown isolates. To test our assumptions, a subset of the A(H3N2) viruses from the 2010–2011 season were studied in detail. Nine (75%) of 12 viruses cultured in MDCK cells for a total of 1 to 3 passages using the standard procedure showed the NA sequence variances at position 148 and/or 151 (Table 2). Their respective clinical specimens were then used to reisolate viruses on both MDCK and

TABLE 2 Substitutions at positions 148 and 151 in the neuraminidase of a subset of A(H3N2) viruses propagated on MDCK cells according to the standard procedure used in routine surveillance

Virus ID[a]	Virus name	GISAID accession no.[b]	Final passage no.	Substitution at residue:	
				148	151
1	A/Alaska/13/2011	EPI438310	1	–[c]	–
2	A/American Samoa/7415/2011	EPI438318	1	T/I[d]	D/N[e]
3	A/American Samoa/7102/2011	EPI438314	1	T/I	D/G
4	A/Vermont/17/2011	EPI438350	2	T/I	–
5	A/Texas/18/2011	EPI438338	2	T/I	D/G/N
6	A/Arizona/07/2011	EPI438322	2	T/I	D/G
7	A/Texas/24/2010	EPI438342	2	–	–
8	A/Pennsylvania/03/2011	EPI438334	2	–	–
9	A/Utah/09/2011	EPI438346	2	T/I	D/G
10	A/New York/11/2011	EPI438330	2	I	–
11	A/Nebraska/12/2011	EPI438326	3	I	–
12	A/Mississippi/05/2011	EPI408578	3	I	–

[a] The virus identifiers (IDs) correspond to those in Table 3 and in Tables S3 and S5 in the supplemental material.
[b] NA gene sequences of viruses in the clinical specimen.
[c] –, no changes observed (wild type).
[d] T/I, mixture of threonine and isoleucine at position 148.
[e] D/N, mixture of aspartic acid and asparagine at position 151.

TABLE 3 Substitutions at positions 148 and 151 in the neuraminidase of a subset of A(H3N2) viruses passaged on MDCK and MDCK-SIAT1 cells using modified procedures

Virus ID	Substitution using modified procedure																			
	MDCK cells										MDCK-SIAT1 cells									
	P1		P2		P3		P4		P5		P1		P2		P3		P4		P5	
	148	151	148	151	148	151	148	151	148	151	148	151	148	151	148	151	148	151	148	151
1	–[a]	–	–	–	–	D/G[b]	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	T/I[c]	–	I	–	I	–	I	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	D/N	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	T/I/K	–	I/K	–	I/K	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	T/I	D/G	T/I	–	I	–	–	–	–	–	–	–	–	–	–	–
8	–	–	–	–	–	–	T/K	–	T/K	–	–	–	–	–	–	–	–	–	–	–
9	–	–	–	–	T/I	–	T/I	–	I	–	–	–	–	–	–	–	–	–	–	–
10	–	–	–	–	–	D/G	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	D/G	–	–	–	–	–	–	–	–	–	–	–	D/G
12	ND[d]	ND																		

[a] No changes observed (wild type).
[b] D/G, mixture of aspartic acid and glycine at position 151.
[c] T/I, mixture of threonine and isoleucine at position 148.
[d] ND, not done; the attempt to reisolate the virus failed.

SIAT1 cells using the modified procedure described in Materials and Methods. Following each passage, the cell culture supernatants were harvested and tested to determine the infectious-virus titers, HA titers, NA sequences at residues 148 and 151, and IC_{50}s for four NA inhibitors in the fluorescent NI assay.

Eleven of 12 viruses were successfully reisolated in both cell lines and were passaged five times (Table 3). The MDCK cell- and SIAT1 cell-grown viruses produced similar infectious-virus titers ranging from 5.9 to 9.2 $\log_{10}$ $TCID_{50}$/ml (see Table S5A and B in the supplemental material). Consistent with our hypothesis, no changes at either residue 148 or 151 were detected in any SIAT1 cell-grown virus, with the exception of the D151G NA substitution (40% by SNP) detected in a single virus (no. 11) after the last passage (Table 3). Notably, the emergence of the NA variants was also delayed when the modified procedure was used to culture the viruses in MDCK cells. After the first passage, no substitutions at either position 148 or 151 were detected in any of the isolates (Table 3). By the second passage, the T148I substitution was detected in a single isolate (no. 2), and the third passage yielded four (36%) viruses with changes at position 148. Peculiarly, the NA variants detected in three P3 and one P4 isolates (no. 1, 3, 10, and 11) were not found after further passaging. After the fifth passage, five isolates contained NA variants at residue T148 but none at residue 151. Two viruses (no. 4 and 5) retained wild-type sequence at both residues 148 and 151 for all passages using the modified procedure, while the same two viruses had changes at residue 148 and/or 151 when propagated under the standard procedure. In some instances, besides the delayed emergence, there was also a difference in the substituted residue. For example, the passaging of two viruses (no. 10 and 11) resulted in the emergence of the T148I variant using the standard procedure, while the D151G variant emerged when culture was performed using a low MOI. To thoroughly compare the P5 isolates to their respective clinical specimens, sequencing was performed, and no additional changes in the NA were detected besides those identified by pyrosequencing.

Overall, after three passages in MDCK cells, a significantly lower number of mutants emerged at both residues T148 ($P = 0.0003$) and D151 ($P = 0.043$) using the modified procedure than using the standard procedure. In the modified procedure, after five passages, a significantly lower number of mutants emerged at residue T148 in MDCK-SIAT1 cells than in MDCK cells ($P < 0.0001$); there was no significant difference in the proportions of mutants that emerged at residue D151 ($P > 0.05$).

To assess the potential effect of virus culture on drug susceptibility, supernatants collected after each passage in both cell lines ($n = 110$) were grouped according to their sequences at residues 148 and 151 and tested in the NI assay. When present either alone or in a mixture with the wild type, the I148 NA variant displayed a slight increase in zanamivir IC_{50}s, although it was not statistically significant (see Table S6 in the supplemental material). The combination of T148K/I was accompanied by the highest increase (7-fold) in zanamivir IC_{50}s. Meanwhile, T148I had no apparent effect on the IC_{50}s for oseltamivir, peramivir, and laninamivir (data not shown).

Despite the overall similarities in the infectious-virus titers, the HA titers of several MDCK cell-grown viruses were greater than those of their SIAT1 cell-grown counterparts (see Table S5A and B in the supplemental material). One SIAT1 cell-grown virus (no. 2) produced no agglutination of guinea pig red blood cells despite the high infectious titers, whereas its MDCK cell-grown counterpart had relatively high HA titers, with the substitution T148I in the NA. With the exception of a single virus (no. 8 contained P221L), no amino acid differences in the HA of the MDCK cell-grown viruses compared to their respective clinical specimens were detected. Therefore, the increase in the HA titers of the MDCK cell-grown viruses often coincided with the emergence of the NA variants (see Table S5A in the supplemental material). This observation supports the proposed role of the 148 substitution in the NA ability to bind to NeuAc-containing receptors.

Properties of recombinant NAs. To ascertain the role of T148I in the observed elevated IC_{50}s, recNA of A/Perth/10/2010—an NA identical to that of A/Mississippi/05/2011 except for their dif-

TABLE 4 IC_{50}s of recombinant neuraminidases tested in the fluorescent NI assay

NA substitution	Mean $IC_{50} \pm SD^a$ [nM (fold^b)]			
	Zanamivir	Oseltamivir	Peramivir	Laninamivir
E119V	0.54 ± 0.13 (3)	44.93 ± 3.76 (281)	0.12 ± 0.02 (1)	0.43 ± 0.08 (4)
T148I	1.33 ± 0.16 (6)	0.04 ± 0.00 (1)	0.06 ± 0.00 (1)	0.34 ± 0.02 (3)
E119V, T148I	220.48 ± 14.00 (1,050)	984.62 ± 76.60 (6,154)	9.54 ± 1.67 (119)	79.47 ± 10.30 (722)
None	0.21 ± 0.03	0.16 ± 0.07	0.08 ± 0.01	0.11 ± 0.04

[a] Based on at least three independent experiments.
[b] Fold change based on comparison to the respective IC_{50} values of recNA E119 and T148 (wild type).

ference at position 464 (I464L)—and its subsequent mutants possessing E119V and/or T148I substitutions were generated and tested in the NI assay. The recNA T148I displayed slightly elevated zanamivir and laninamivir IC_{50}s—6- and 3-fold, respectively—and unaltered IC_{50}s for the other two NA inhibitors (Table 4). As anticipated, the recNA E119V showed a 281-fold increase in the oseltamivir IC_{50}, whereas the IC_{50}s of the other three NA inhibitors were either unchanged or slightly elevated (1- to 4-fold). Notably, the recNA E119V-T148I exhibited highly elevated IC_{50}s of all four NA inhibitors, ranging from 119- to 6,154-fold, depending on the drug (Table 4). The T148I substitution also caused a 50% reduction in NA activity, and the detrimental effect was even greater (loss of 96% of activity) when this substitution was combined with E119V (data not shown).

DISCUSSION

In the present study, we demonstrate that culturing influenza A(H3N2) viruses in MDCK cells can alter their drug susceptibility phenotypes. The oseltamivir-resistant A(H3N2) E119V virus was identified among viruses submitted for virological surveillance, and when tested in the NI assay, its P3 isolate displayed cross-resistance to four NA inhibitors tested (oseltamivir, zanamivir, peramivir, and laninamivir). This unusual observation prompted us to further investigate the properties of the virus and led to discovery of the role played by the T148I substitution identified in its NA. Unlike the oseltamivir-selected E119V, T148I was not detected in the clinical specimen and was shown to be a consequence of the virus propagation in MDCK cells.

Substitutions at two other residues, 136 and 151, in the NA of A(H1N1) have been shown to affect drug NA inhibitor susceptibility and were linked to influenza virus propagation in MDCK cells (31, 32). Moreover, previous studies have shown that the MDCK cell-grown A(H3N2) virus contained substitutions at positions 148 and 151, whereas its counterpart propagated in CaCo-2 cells did not have such changes (10). Despite the growing evidence regarding selection of NA variants in MDCK cells, the cell line continues to be the most common substrate for the propagation of human influenza viruses.

In this study, two simple approaches to mitigate the emergence of NA variants during culture were explored. Propagation of A(H3N2) viruses was conducted at a low MOI (modified procedure) on both MDCK and SIAT1 cells. Experimental evidence that the low MOI (0.001) was sufficient to delay the NA variant emergence on MDCK cells was provided. The result was even better if isolation and propagation were carried out on SIAT1 cells. However, concerns have arisen that SIAT1 cells may select for HA variants of A(H1N1)pdm09 viruses with altered antigenic properties and thus may not be a suitable culture for propagation of all influenza viruses (33). For the set of A(H3N2) viruses analyzed in this study, similarly high infectious titers (ranging from 5.9 to 9.2 $\log_{10}$ $TCID_{50}$/ml) were achieved in both cell lines, and the NA activities were sufficient to determine drug susceptibilities in the NI assay. However, the HA titers of certain SIAT1 cell-grown viruses were lower than those of the MDCK cell-grown viruses, which may preclude their testing in the HI assays. Notably, MDCK cell-grown viruses that acquired changes at position 148 displayed HA titers higher than those of their SIAT1 cell-grown counterparts. This observation indirectly supports the role of this substitution in the increased NA binding to NeuAc-containing receptors. Unlike the D151G substitution (24), the mechanism by which the T148I substitution may increase NA binding to NeuAc-containing receptors is unknown. The T148I change not only introduces a hydrophobic residue that could structurally affect the nearby active site, it also results in the loss of a potential glycosylation site at N146. Interestingly, alignment of contemporary NA sequences from the GISAID database revealed no virus with a substituted N146.

Further experimental evidence was obtained with the use of the recNA proteins. The recNA carrying both E119V and T148I changes showed highly reduced inhibition by all NA inhibitors and a drastic loss of NA activity. T148I alone was associated with a slight increase (6-fold) in the zanamivir IC_{50} while showing little or no effect on inhibition by the other three NA inhibitors; it caused a loss of 50% of NA activity toward MUNANA. NA inhibitors compete with NeuAc-containing receptors for binding to the NA active site. Zanamivir was designed using a minimalistic approach (34) and most closely resembles the structure of NeuAc. It is not surprising, therefore, that zanamivir binding was affected by the T148I substitution.

NA variants with changes at positions 148 and 151 often present as a mixture with the wild type, and their presence could be underreported if conventional sequencing was employed. In this regard, the pyrosequencing assay used in this study offers a superior means to detect and identify minor subpopulations, although it requires highly skilled technical personnel (35).

Our results support the notion that culture of A(H3N2) viruses needs to be carried out at a low MOI whenever possible, and the number of passages should be kept to a minimum. This rule needs to be applied when analyzing samples collected from NA inhibitor clinical trials or when preparing reference viruses used for validation of NI assays. For example, the T148I substitution (~43% by SNP pyrosequencing) was detected in the CDC reference drug-sensitive A/Washington/01/2007 A(H3N2) virus used in this study. Additional efforts should be made to identify suitable cell cultures that do not exert selective pressure on human influenza

viruses that may lead to altered antigenic and drug resistance phenotypes.

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