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The effect of the MDCK cell selected neuraminidase D151G mutation on the drug susceptibility assessment of influenza A(H3N2) viruses

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Abstract

Propagation of influenza A(H3N2) viruses in MDCK cells has been associated with the emergence of neuraminidase (NA) variants carrying a change at residue 151. In this study, the pyrosequencing assay revealed that ~90% of A(H3N2) virus isolates analyzed (n = 150) contained more than one amino acid variant (D/G/N) at position 151. Susceptibilities of the virus isolates to zanamivir and oseltamivir were assessed using the chemiluminescent and fluorescent NA inhibition (NI) assays. In the chemiluminescent assay, which utilizes NA-Star® substrate, up to 13-fold increase in zanamivir-IC_{50} was detected for isolates containing a high proportion (>50%) of the G151 NA variant. However, an increase in zanamivir-IC_{50}s was not seen in the fluorescent assay, which uses MUNANA as substrate. To investigate this discrepancy, recombinant NAs (rNAs) were prepared and tested in both NI assays. Regardless of the assay used, the zanamivir-IC_{50} for the rNA G151 was much greater (>1500-fold) than that for rNA D151 wild-type. However, zanamivir resistance conferred by the G151 substitution was masked in preparations containing the D151 NA which had much greater activity, especially against MUNANA. In conclusion, the presence of NA D151G variants in cell culture-grown viruses interferes with drug susceptibility assessment and therefore measures need to be implemented to prevent their emergence.

Keywords

Neuraminidase inhibition assay; Pyrosequencing; Recombinant protein; NA-Star® kit; NA-Fluor™ kit

Drug susceptibility assessment is an integral part of influenza virological surveillance. Viruses propagated in cell culture (e.g. Madin-Darby canine kidney, MDCK cells) are analyzed in the NA inhibition (NI) assay supplemented with NA sequence analysis (Nguyen et al., 2012a). Two NI assays, chemiluminescent (CL) and fluorescent (FL), are commonly

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used for assessment of virus susceptibility to NA inhibitors (WHO, 2012). Both assays utilize small synthetic substrates, NA-Star® and MUNANA, respectively. Drug susceptibility assessment is based on determination of an IC_{50} value (drug concentration needed to inhibit NA activity by 50%) and its comparison to a reference IC_{50} value (WHO, 2012). Culturing influenza viruses in MDCK cells, especially those of the A(H3N2) subtype, has been linked to the emergence of viruses with a substitution at residue 151 in the NA enzyme active site (Lin et al., 2010; Okomo-Adhiambo et al., 2010; Sheu et al., 2008; McKimm-Breschkin et al., 2003). Lin et al. (2010) tested a reverse genetically engineered A(H3N2) virus carrying the D151G substitution and reported no effect on the NA's ability to cleave MUNANA. However, recombinant NA (rNA) carrying the D151G substitution has been shown to cause reduced enzyme activity resulting in an increased affinity for neuraminic acid-containing receptors (Zhu et al., 2012a). The presence of D151G has occasionally been linked to an elevated IC_{50} for NA inhibitors zanamivir and/or oseltamivir (McKimm-Breschkin et al., 2003; Okomo-Adhiambo et al., 2010; Sheu et al., 2008). Nevertheless the question regarding its effect on drug susceptibility assessment has not been fully addressed.

In the present study, we investigated the effect of changes at D151 in the NA on susceptibilities of MDCK-grown A(H3N2) viruses to zanamivir and oseltamivir in the CL and FL NI assays (Okomo-Adhiambo et al., 2010; Nguyen et al., 2010).

First, we determined the frequency of changes at residue 151 in the NA of MDCK-grown A(H3N2) virus isolates. Based on conventional Sanger sequencing, a single nucleotide polymorphism was commonly observed at the first (G/A) and the second (A/G) nucleotides of the triplet (GAT) which encodes aspartic acid at position 151. Such nucleotide sequence could be interpreted as a mixture of NA variants with aspartic acid (GAT), glycine (GGT), asparagine (AAT) or serine (AGT) at residue 151. To improve the resolution of amino acid variance, we used the pyrosequencing assay with a customized nucleotide dispensation (Table 1, legend).

Next, the NA sequences of 150 A(H3N2) virus isolates collected during 2011–2012 season were determined using this pyrosequencing approach. Three NA variants, aspartic acid (wildtype), glycine and asparagine, were identified, while serine was not detected. Majority of the isolates (133/150, 88.7%) contained a mixture of NA variants (Table 1). Noteworthy, no such variance was detected in matching clinical specimens (0/50, 0%).

All 150 virus isolates were divided into four groups according to their NA sequence at position 151. Their IC_{50} values for zanamivir and oseltamivir were determined in the CL NI assay and mean IC_{50}s were calculated for each group (Table 1). No difference in oseltamivir-IC_{50}s was detected for any of the four groups, while two groups, containing the G151 NA variant, showed a slight increase (~2–4-fold) in zanamivir-IC_{50}s (Table 1). A closer look at the effect of the G151 on zanamivir-IC_{50}s revealed that the viruses (n = 11) with the highest proportion of G151 (50–77%) exhibited on average, 7-fold higher zanamivir-IC_{50} (3.54 ± 1.34 nM). Peculiarly, visual inspection of the NA inhibition curves of the virus isolates containing D/G mixtures revealed the presence of a small 'hump' at the higher (10–300 nM) concentrations of zanamivir, which was not seen in the wild-type virus curve (Fig. 1A and B). Based on these observations, we assumed that the appearance

of the ‘hump’ was caused by the presence of the zanamivir-resistant variant, G151, and that its NA activity must be significantly reduced in comparison to that of the wild-type. On the contrary, the presence of N151 in the virus isolate produced no effect on susceptibility to zanamivir.

Testing this hypothesis required the separation of the NA virus variants, however, several attempts of their plaque purification failed due to re-emergence of the wildtype virus (data not shown). Therefore, a baculovirus expression system was used (Zhu et al., 2012b) to produce recombinant NA (rNA) proteins containing either D, G or N at residue 151.

While no change was observed for the rNA N151 (Fig. 2C), the rNA G151 showed a 1675-fold increase in zanamivir-IC_{50} compared to that of the rNA D151 when tested in the CL NI assay (Table 2; Fig. 2A and B). Moreover, comparison of the rNA preparations, normalized by protein content, showed that the D151G substitution caused a 5-fold reduction in the enzyme’s ability to process the NA-Star® substrate (Fig. 3A).

In the next experiment, the rNA D151 and rNA G151 preparations were mixed at different proportions and the mixtures were tested in the CL NI assay. The inhibition curves of the 1:1 (G151 = 50%) and 1:3 (G151 = 75%) mixtures had the same characteristic ‘hump’ (Fig. 2D and E) previously observed for the MDCK-grown isolates (Fig. 1B). In addition, a slight increase (2-fold) in zanamivir-IC_{50}s was observed (Table 2). The mixture with the highest G151 content (G151 = 90%) showed a 16-fold increase in zanamivir IC_{50} (Table 2) and its inhibition curve had an even larger ‘hump’ (Fig. 2F).

When the rNAs were tested in the FL NI assay, the rNA G151 exhibited highly elevated (1804-fold) zanamivir-IC_{50} (Table 2), which was similar to the result of the CL NI assay. In contrast, no increase in IC_{50}s was observed when the mixtures of the rNA D151 and rNA G151 were tested (Table 2). This difference was explained by the highly reduced ability (~100-fold) of the rNA G151 to cleave MUNANA compared to the cleavage of NA-Star® substrate (Fig. 3B).

The use of rNAs provided a valuable option to study effects of changes at D151 on enzyme activity and its inhibition by antiviral drugs. This approach offers an advantage over the use of reverse genetically engineered viruses which need to be propagated in cell culture prior to testing and thus are subject to selective pressures.

Influenza virus variants carrying NA substitutions which emerge in response to drug treatment are commonly present together with the wild-type viruses (McKimm-Breschkin, 2005). Compared to the CL, the FL NI assay was shown to be more sensitive in detecting oseltamivir-resistant viruses with the H275Y substitution when present as a mixture with wild-type A(H1N1)pdm09 viruses (Nguyen et al., 2010, 2012b; Hurt et al., 2012). In contrast, as shown in the present study, the CL NI assay is superior in detecting the zanamivir-resistant A(H3N2) viruses carrying D151G. These findings indicate that NA variants’ ability to process the synthetic substrate used in an NI assay, can affect the assay’s sensitivity in detecting drug-resistant variants present in a mixture with wild type viruses.

The NA substitution D151G arises during passaging of contemporary A(H3N2) viruses in MDCK cells. As shown in this study, this substitution confers resistance to zanamivir, but has no effect on oseltamivir susceptibility. In laboratories utilizing FL NI assay, the zanamivir resistance conferred by this change typically goes unnoticed; however, the users of CL NI assay need to be aware of an increase in zanamivir-IC_{50}s which should be interpreted as an artifact of influenza virus propagation in MDCK cells. To assure rapid and accurate evaluation of properties of this continuously evolving human pathogen, a spectrum of assays and technologies need to be available to public health laboratories within the WHO Global Influenza Surveillance and Response System (GISRS).

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Disclaimer

We declare that we have no potential conflicts of interest. 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 (CDC).

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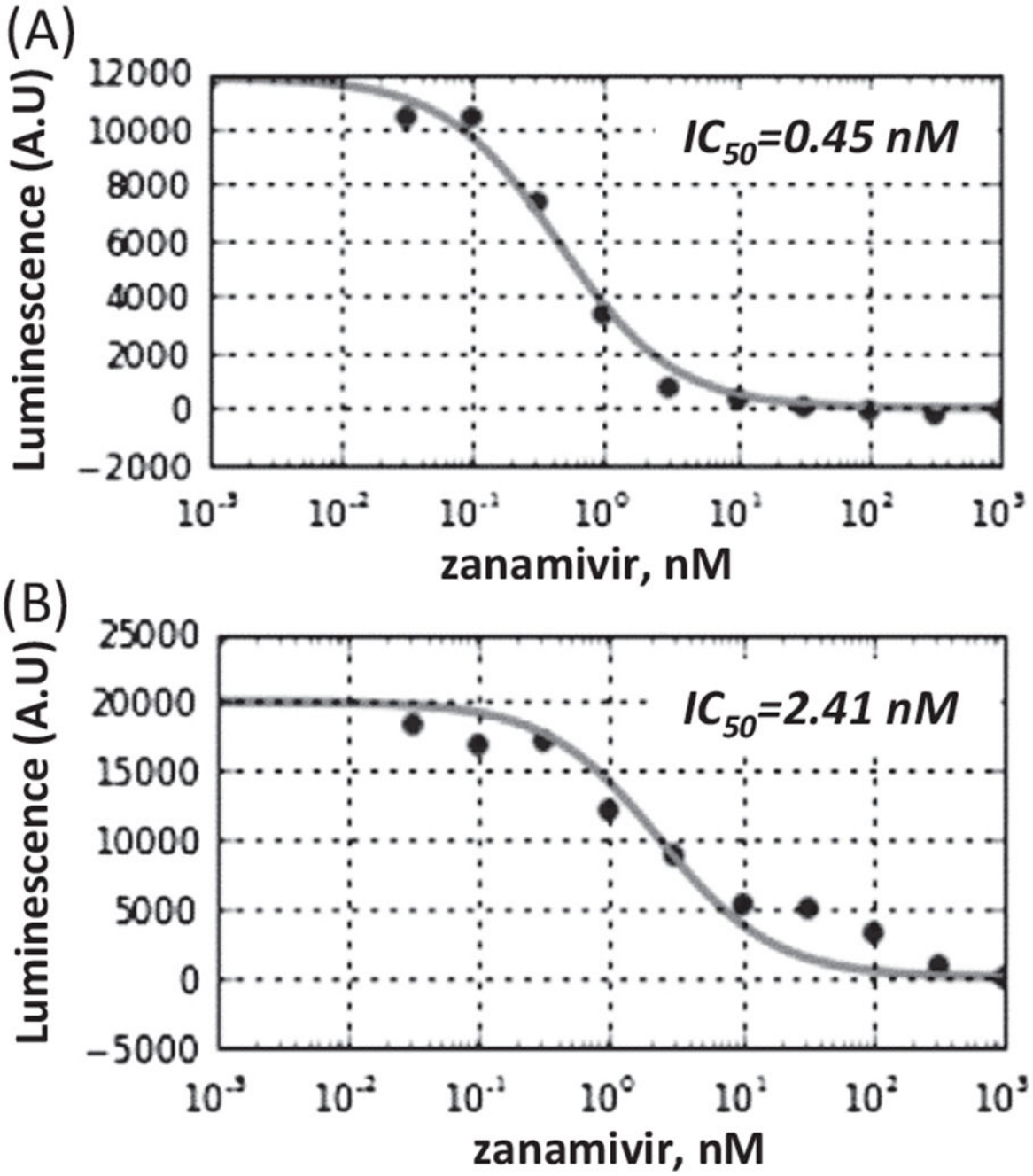

Fig. 1.

Inhibition curves for the MDCK-grown A(H3N2) viruses tested in the CL NI assay. (A) The wild-type virus isolate (D151) exhibiting a typical S-shaped curve for inhibition of NA enzyme activity at increasing concentrations of zanamivir; (B) the virus isolate containing a 1:1 mixture of the D151 and G151 NA variants exhibiting a flattened S-curve with the characteristic ‘hump’ at the higher concentrations of zanamivir.

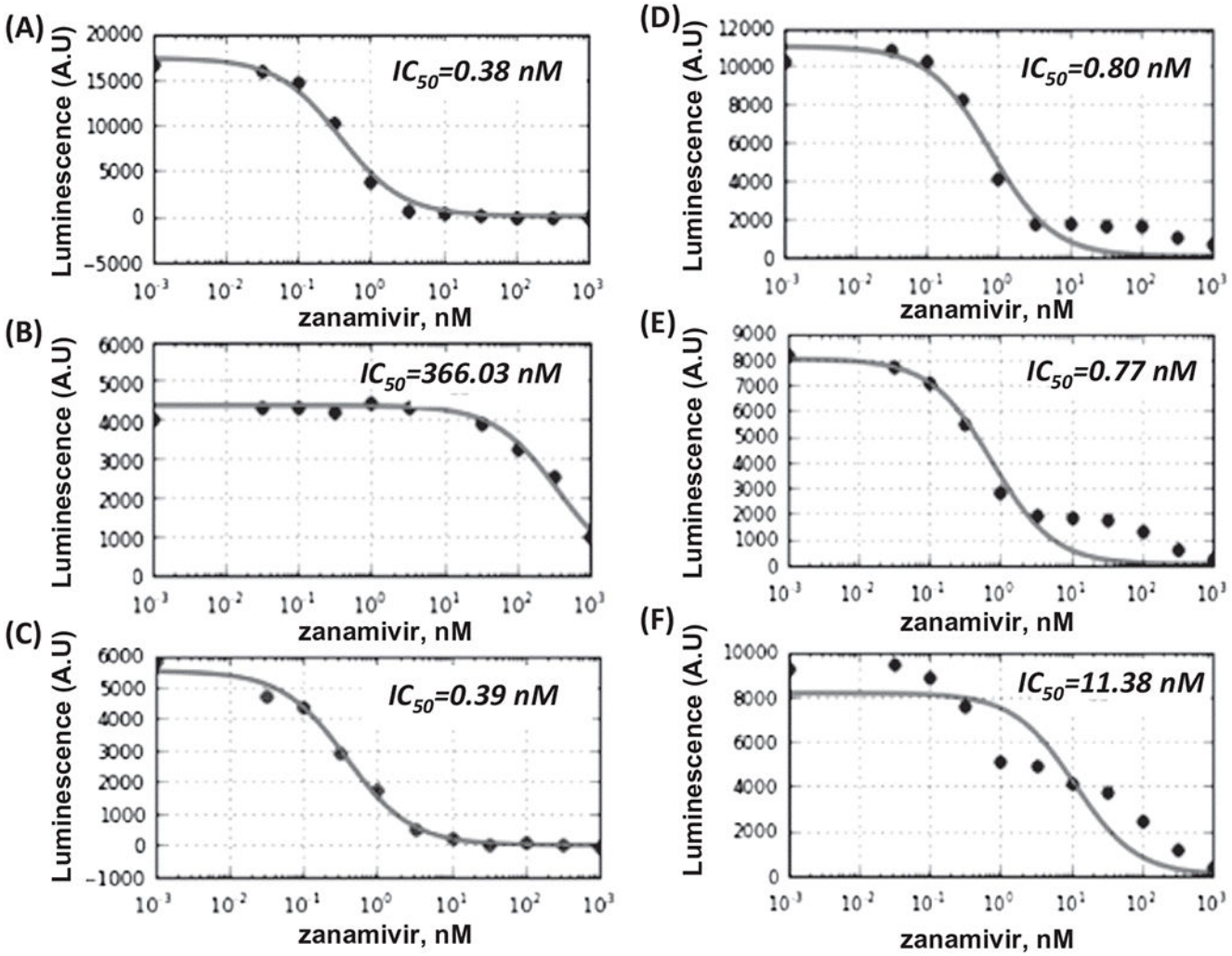

Fig. 2.

Inhibition curves for rNAs tested in the CL NI assay. (A) rNA-D151; (B) rNA-G151; (C) rNA-N151; (D) 1:1 mixture of rNA-D151 and rNA-G151; (E) 1:3 mixture of rNA-D151 and rNA-G151; (F) 1:9 mixture of rNA-D151 and rNA-G151.

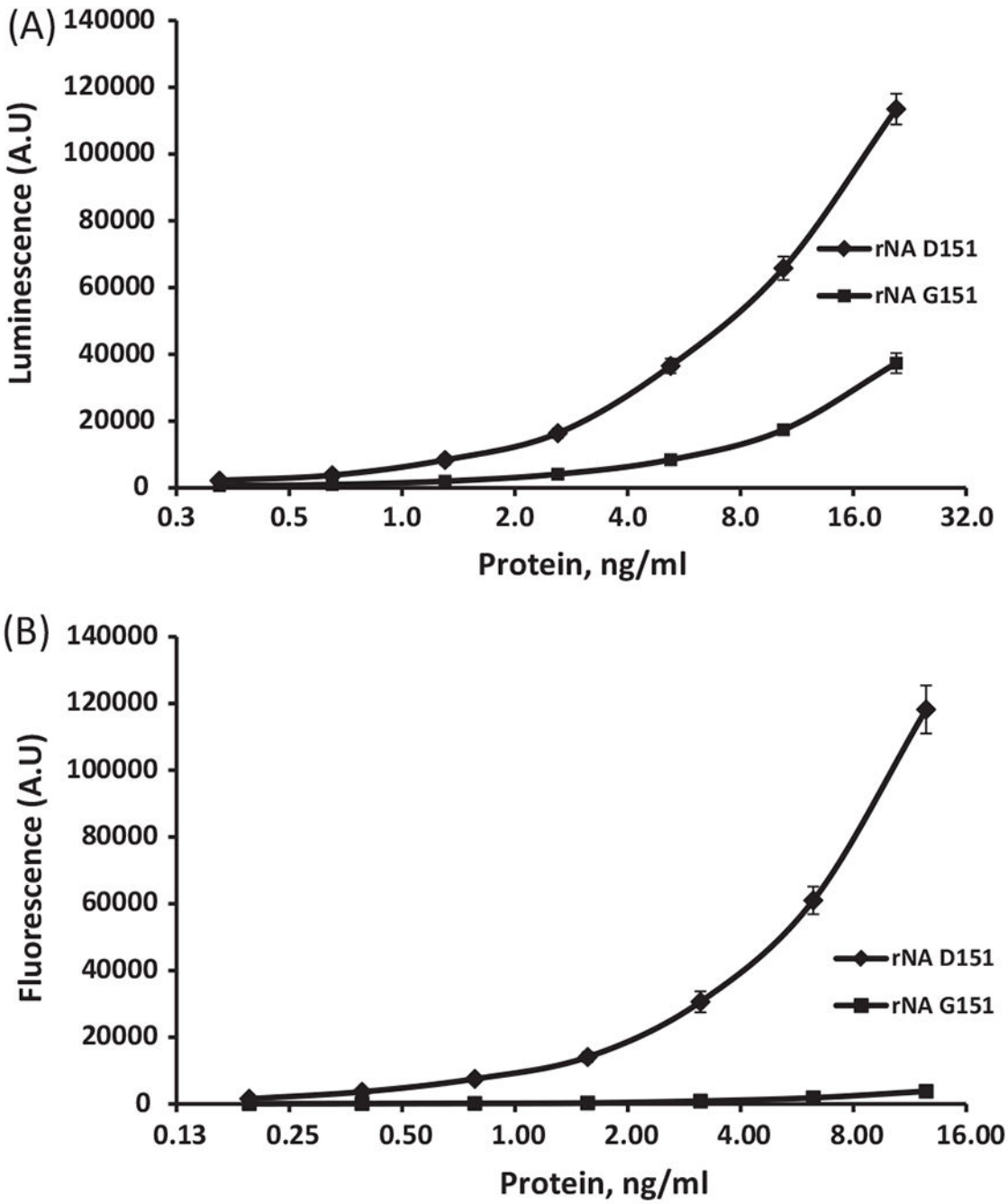

Fig. 3.

Comparison of the NA activity of the two rNAs, D151 and G151, in the CL (A) and FL (B) NA assays. The NA gene of A/Perth/16/2009 (H3N2) was cloned, expressed in a baculovirus expression system and purified, as previously described (Zhu et al., 2012b). The concentration of rNA present in the preparations was determined using the Bradford method by comparison with serially diluted bovine serum albumin (BSA). rNAs preparations were

normalized by protein content and 2-fold serial dilutions were prepared and tested for NA activity using CL or FL NA assays. Vertical error bars are showing standard deviation (SD).

Table 1

Characterization of MDCK-grown A(H3N2) virus isolates using pyrosequencing and CL NI assay.

Group	Variance at 151[a]	No. viruses	IC_{50}, nM (fold)[b]	
			Zanamivir	Oseltamivir
1	D	17	0.48 ± 0.14	0.13 ± 0.08
2	D/N	34	0.66 ± 0.22 (1)	0.16 ± 0.07 (1)
3	D/G/N	57	1.14 ± 1.16 (2)	0.16 ± 0.08 (1)
4	D/G	42	1.93 ± 1.90 (4)[c]	0.17 ± 0.07 (1)

[a] Amino acid sequence variance at residue 151 in the NA of 150 virus isolates was determined using the pyrosequencing assay. The SQA mode with the customized nucleotide dispensation (TACGCTAGCAGTATCGTCTATCA[GATC]5) was used. The primers used for RT-PCR amplification of the NA gene fragment were designed using PSQ Assay Design software v.1.0.6 (Qiagen); the forward primer N2NA151-F423 (5′-CAACGTGCATTCAAATGACAC-3′) and the reverse primer N2NA151-R567b (biotin-5′-CC AYG CTT TYC CAT CRT G-3′). The forward primer was used to perform the pyrosequencing reaction. The limit of detection was set at 10%. Pyrogram analysis was done, essentially as described previously (Levine et al., 2011). Detailed protocol is available upon request.

[b] Mean ± SD, fold increase compared to the mean IC_{50} of D151 viruses.

[c] Statistically significant ($p < 0.05$) difference compared to the mean zanamivir IC_{50} of D151 viruses (wild type).

Table 2

Inhibition of enzyme activity of the rNA proteins in CL and FL NI assays.

NI assay	rNA (ratio)[a]	IC_{50}, nM (fold)[b]	
		Zanamivir	Oseltamivir
CL	D151	0.44 ± 0.05	0.21 ± 0.02
	G151	742.94 ± 137.9 (1675)	0.18 ± 0.08 (1)
	D151:G151 (1:1)	0.82 ± 0.02 (2)	0.19 ± 0.05 (1)
	D151:G151 (1:3)	0.68 ± 0.09 (2)	0.18 ± 0.04 (1)
	D151:G151 (1:9)	6.92 ± 3.17 (16)	0.34 ± 0.06 (2)
FL	D151	0.33 ± 0.19	0.5 ± 0.14
	D151G	590.95 ± 201.1 (1804)	0.49 ± 0.01 (1)
	D151:G151 (1:1)	0.39 ± 0.05 (1)	0.4 ± 0.10 (1)
	D151:G151 (1:3)	0.40 ± 0.04 (1)	0.40 ± 0.02 (1)
	D151:G151 (1:9)	0.42 ± 0.05 (1)	0.40 ± 0.06 (1)

[a] Preparations of rNA D151 and rNA G151 were normalized by protein content and mixed at ratios 1:1, 1:3 or 1:9 (also see legend to Fig. 3).

[b] Mean ± SD, fold increase compared to the mean IC_{50} of the rNA D151.