



The 2008–2009 H1N1 influenza virus exhibits reduced susceptibility to antibody inhibition: Implications for the prevalence of oseltamivir resistant variant viruses

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ABSTRACT

A naturally-occurring H275Y oseltamivir resistant variant of influenza A (H1N1) virus emerged in 2007, subsequently becoming prevalent worldwide, via an undetermined mechanism. To understand the antigenic properties of the H275Y variant, oseltamivir resistant and susceptible strains of H1N1 viruses were analyzed by hemagglutination inhibition (HI) and microneutralization assays. HI analysis with H1-positive sera obtained from seasonal flu vaccine immunized and non-immunized individuals, and H1-specific monoclonal antibodies, revealed that resistant strains exhibited a reduced reactivity to these antisera and antibodies in the HI assay, as compared to susceptible strains. Neutralization assay testing demonstrated that oseltamivir resistant H1N1 strains are also less susceptible to antibody inhibition during infection. Mice inoculated with a resistant clinical isolate exhibit 4-fold lower virus-specific antibody titers than mice infected with a susceptible strain under the same conditions. Resistant and sensitive variants of 2009 pandemic H1N1 virus did not exhibit such differences. While HA1 and NA phylogenetic trees show that both oseltamivir resistant and susceptible strains belong to clade 2B, NA D354G and HA A189T substitutions were found exclusively, and universally, in oseltamivir resistant variants. Our results suggest that the reduced susceptibility to antibody inhibition and lesser *in vivo* immunogenicity of the oseltamivir resistant 2008–2009 H1N1 influenza A virus is conferred by coupled NA and HA mutations, and may contribute to the prevalence of this H1N1 variant.

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1. Introduction

Resistance to rimantadine and amantadine, viral M2 ion channel blockers, has been commonly observed in H3N2 influenza viruses since 2003 and also in the 2009 pandemic H1N1 virus (Bright et al., 2005, 2006; Garten et al., 2009); as such, treatment of influenza virus infection has relied mainly on neuraminidase (NA) inhibitors, which inhibit virus release from infected cells by targeting viral NA. The NA inhibitors oseltamivir and zanamivir have been available for treatment of influenza since 1999, while another NA inhibitor, peramivir, was recently registered for use in Japan (Kohno et al., 2011; Sugaya, 2011; Zambon and Hayden, 2001). Surveillance studies facilitated via the global neuraminidase inhibitor

susceptibility network (NISN) found that resistance to each neuraminidase inhibitor was rare during the first few years of clinical use (Hurt et al., 2009b; Kiso et al., 2004; McKimm-Breschkin et al., 2003; McKimm-Breschkin, 2000; Monto et al., 2006). It was also shown that resistant mutants obtained through selection in culture or isolated from patients treated with neuraminidase inhibitors are subtype specific, with E119V and R292K NA mutations occurring mainly in H3N2 subtype viruses exposed to either oseltamivir or zanamivir, and the H275Y mutation in the NA protein found almost exclusively among H1N1 subtype viruses in response to oseltamivir treatment (McKimm-Breschkin et al., 2003; McKimm-Breschkin, 2000; Sheu et al., 2008; Yen et al., 2006; Zambon and Hayden, 2001). Further characterization revealed that those non-naturally occurring resistant mutants selected by exposure to oseltamivir or zanamivir have compromised infectivity and replication abilities (Bouvier et al., 2008; Carr et al., 2002; Herlocher et al., 2002, 2004; Ives et al., 2002; Yen et al., 2005).

Resistance to oseltamivir (Tamiflu) has been monitored extensively as it is used more commonly than zanamivir and peramivir

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due to its oral availability, and is one of the most stockpiled antiviral drugs for the preparedness for a potential pandemic, as recommended by the World Health Organization (WHO) (Oxford, 2005; Reddy, 2010). Prior to 2007, only a very small number of H275Y mutant oseltamivir resistant viruses were recognized, mainly clinical isolates following oseltamivir treatment (Hurt et al., 2009b). However, the emergence of a H275Y resistant H1N1 variant in patients without pre-exposure to oseltamivir was identified in Norway in 2007 (Hauge et al., 2009). Similar resistant isolates were subsequently found in other countries and these rapidly replaced susceptible strains around the world (Cheng et al., 2009; Dharan et al., 2010; Weinstock and Zuccotti, 2009). The mechanism for the emergence and prevalence of this H275Y variant is not fully understood. In contrast to the resistant isolates identified before 2007, which had compromised growth and infection abilities, this naturally occurring resistant H1N1 strain appeared to cause illness similar to that associated with the wild type virus (Dharan et al., 2009; Hauge et al., 2009). The H275Y variant was found to replicate and transmit similarly to wild type virus in cells and in a competitive mixture model, respectively (Hurt et al., 2010). Nevertheless, it is still not clear how this resistant variant managed to replace the wild type virus and become the predominant population in humans in a relatively short period of time in the apparent absence of drug selection pressure. Resistance to oseltamivir has been detected in some clinical isolates of the pandemic H1N1 virus from patients both with and without a history of exposure to this drug (Chen et al., 2009; Hurt et al., 2011; Meijer et al., 2011). There is a concern that the resistant variant of the 2009 pandemic H1N1 virus may evolve to replace susceptible strains as the dominant population.

Host immunity gained through vaccination or previous infection plays an important role in determining the extent of prevalence of a strain of influenza virus. Influenza viruses undergo continuous antigenic drift to evade existing host immunity. It is believed that the ability of a new virus to rise to prevalence in a population is jointly determined by viral elements which enable robust replication in human tissues and efficient transmission between humans, together with levels of host immunity in the human population. This study compared the antigenic properties of oseltamivir resistant variant and wild type H1N1 viruses, analyzing the reactivity of antisera obtained from individuals who had received seasonal vaccine and from members of the general population who had no history of influenza vaccination to the viruses. We found that 2008–2009 H1N1 influenza A viruses which are resistant to oseltamivir exhibit different antigenic and immunogenic features than the wild type virus, which may contribute to the altered sensitivity of resistant virus strains to neutralizing antibodies.

2. Materials and methods

2.1. Viruses and antibodies

Clinical isolates of oseltamivir resistant and susceptible H1N1 viruses were kindly provided by Dr. W. Lim of the Centre for Health Protection, Hong Kong SAR. The identity of residue 275 of the NA gene was confirmed by sequencing analysis for all viruses used (see Supplementary Table 1 for GenBank accession numbers). Recombinant viruses carrying HA and NA from an oseltamivir resistant seasonal H1N1 influenza virus (A/HK/62768/2008), with the other six segments derived from A/Puerto Rico/8/1934 (PR8) virus, were constructed using the pHW2000 system, as described previously (Wang et al., 2009). Briefly, the HA and NA of A/HK/62768/2008 were cloned into the pHW2000 vector (Hoffmann et al., 2000). A Y275H substitution was introduced into the NA segment

using the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. Recombinant viruses carrying either wild type NA (RG62768 NA275Y) or mutated NA (RG62768 NA275H) plus HA from A/HK/62768/2008 and the remaining six segments from PR8 were rescued. All gene segments from both recombinant viruses were sequenced to confirm the absence of undesired mutations.

Immunized antisera were obtained from individuals (aged from 20 to 50 years old) who received 2008–2009 seasonal trivalent influenza vaccine, which is composed of the A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B/Florida/4/2006-like virus strains. H1-reactive antisera (176) were identified by screening sera from 1192 individuals from the general population who had not had the influenza vaccine for reactivity with A/Brisbane/59/2007. Murine monoclonal antibodies to A/Brisbane/59/2007 and A/California/7/2009 2009 pandemic H1N1 virus were raised using procedures similar to those previously described (Wu et al., 2008). Briefly, inactivated H1N1 viruses (adjusted to a titer of 5×10^7 TCID₅₀/ml before inactivation) were emulsified with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, USA) and injected subcutaneously into six-week-old female BALB/c mice at two weekly intervals. A final dose of 100 µl of inactivated virus was injected intravenously 3 days prior to the procedure by which spleen cells from the immunized mice were fused with mouse myeloma cells (SP2/O-Ag14). Hybridomas were screened for the secretion of H1-specific MAbs by HI assay. MAbs were prepared by injecting hybridoma cultures into the peritoneal cavities of pristine-primed BALB/c mice; the ascitic fluid was collected after 9–12 days and stored at -20°C . MAbs were purified using ammonium sulfate precipitation, followed by DE-52 ion exchange chromatography. Immunoglobulin (Ig) concentrations were determined by spectrophotometry using a Perkin Elmer MBA 2000 spectrometer.

2.2. Hemagglutination inhibition and microneutralization assays

HI tests and microneutralization assays were performed in accordance with the World Health Organization guidelines (World Health Organization, 2002). Turkey red blood cells (RBC) were used for the standard HI assays. All polyclonal antisera were treated with sialic acid receptor destroying enzyme (RDE, Denka Seiken Co., Tokyo, Japan) prior to testing. HI test serial dilutions were started at 1:10 dilution for antisera and 1:100 dilution for monoclonal antibodies, as previously described (Wu et al., 2008). To determine the effect of neuraminidase activity on the HI assay, viruses were treated with zanamivir (RELENZA, GlaxoSmithKline, UK) prior to the addition of antisera. Briefly, 8 HA units of virus were mixed with 5 nM zanamivir at a 1:1 (v/v) ratio and incubated at room temperature for 1 h, then used for HI testing.

2.3. In vitro growth kinetics

Growth kinetics were studied using procedures previously described (Wu et al., 2008). Briefly, 90% confluent MDCK or A549 cells (ATCC, Manassas, USA) were infected with H1N1 viruses at a multiplicity of infection (MOI) of 0.1 for 1 h at 37°C . Infected cells were incubated in serum free medium in a 5% CO₂ atmosphere at 37°C . At 4, 8, 12, 24 and 36 h post infection (pi), the supernatants of the infected cells were harvested and plaque assays were conducted in MDCK cells to determine virus titers.

2.4. Enzymatic analysis

K_M and V_{max} values of neuraminidases were estimated using purified virus particles, as previously described (Potier et al., 1979). Briefly, the 2008–2009 H1N1 viruses were cultured in

MDCK cells and supernatants were cleared by centrifugation at 5000 rpm for 15 min in JLA16.250 rotor (Beckman-Coulter) and the viruses were pelleted at 40,000 rpm for 1 h at 4 °C in a 45Ti rotor (Beckman-Coulter) and re-suspended in Tris-HCl buffer for enzymatic analysis. NA enzymatic activity was measured using fluorogenic substrate (MUNANA; Sigma). The fluorescence of released 4-methylumbelliferone was measured at excitation and emission wavelengths of 330 and 450 nm, respectively. The enzyme kinetics data were fitted to the Michaelis-Menten equation by using nonlinear regression (Prism; GraphPad) to determine the Michaelis constant (K_M) and maximum velocity (V_{max}) of substrate conversion.

2.5. Phylogenetic and sequence analyses

HA and NA genes from oseltamivir resistant and susceptible strains of 2008–2009 H1N1 virus were sequenced using standard procedures. Sequence assembly, alignment, and residue analysis of HA and NA genes were performed as previously described (Chen et al., 2006). The phylogenetic trees of nucleotide sequences were analyzed with PAUP* software, using the neighbor-joining method with 1000 bootstraps.

2.6. Preparation of antisera from infected mice

Antibody responses in mice were tested by infecting 6- to 8-week-old female BALB/c mice with H1N1 viruses. Oseltamivir resistant and susceptible strains of 2008–2009 H1N1 and 2009 pandemic H1N1 viruses and recombinant 275H and 275Y versions of one of the 2008–2009 H1N1 viruses were used in the experiment. All procedures were conducted in a Biosafety Level 3 facility at the Department of Microbiology, The University of Hong Kong. Mice were lightly anaesthetized by inhalation of 4% isoflurane (Halocarbon Laboratory, River Edge, NJ, USA) and inoculated intranasally with 25 μ l PBS containing $10^{4.5}$ TCID₅₀ of H1N1 virus. Blood was collected at 21 days post infection and sera were treated with RDE prior to HI testing.

3. Results

3.1. Antigenic analysis of 275Y and wild type 275H isolates with antisera and H1 specific monoclonal antibodies

It has been reported previously that the oseltamivir resistant H1N1 viruses containing H275Y mutant NA replicate similarly to wild type viruses in cell culture using canine MDCK and MDCK-SIAT1 cells (Hurt et al., 2010). We confirmed that there is no statistically significant difference in growth kinetics between oseltamivir resistant and susceptible strains in the human cell line, A549 (Supplementary Fig. 1).

To investigate if oseltamivir resistant H1N1 viruses may have other distinct properties, we examined whether the 275Y strain might be equipped with an enhanced ability to escape host immunity, making it more likely to be shed and transmitted between humans. We first conducted an antigenic analysis using a panel of antisera collected from 20 individuals who had received seasonal trivalent influenza vaccine containing the H1N1 vaccine strain A/Brisbane/59/2007, which is an oseltamivir susceptible strain. The level of antibodies to A/Brisbane/59/2007 in these immunized sera were confirmed to be increased at least 4-fold, compared to unimmunized sera, by HI assay (data not shown). As demonstrated in the reverse cumulative distribution hemagglutination inhibition titer curves, the antisera reacted less strongly with the resistant 275Y strains ($N=21$) as compared to the susceptible 275H strains ($N=10$) (Fig. 1A, Supplementary Table 1A). To further test if

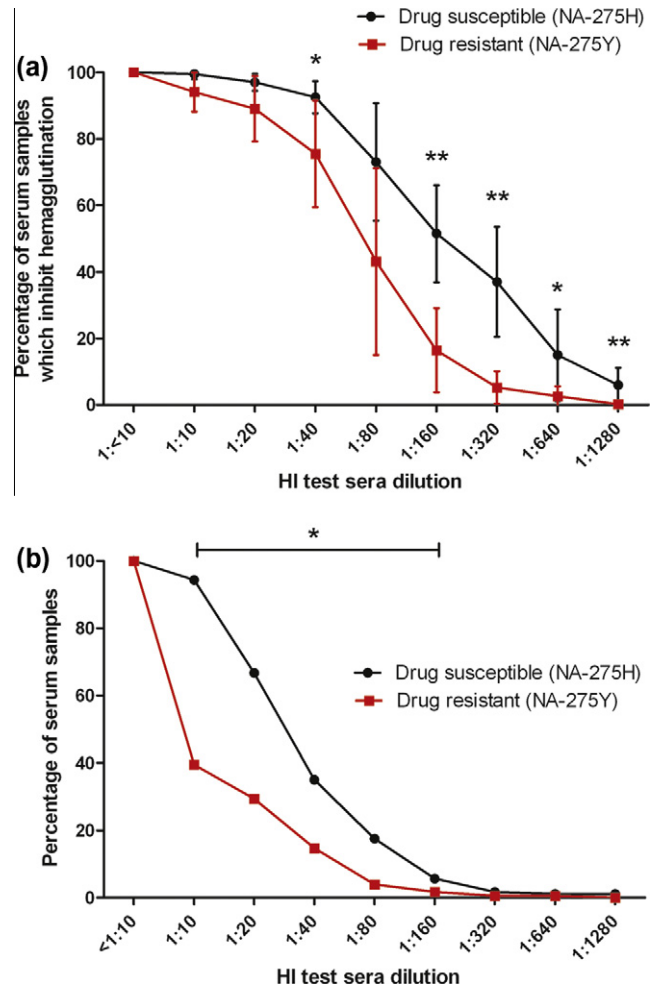


Fig. 1. Comparison of hemagglutination-inhibition activity of antisera against NA 275H and 275Y H1N1 clinical isolates. (A) HI titers were estimated for 20 antisera obtained from individuals immunized with seasonal trivalent vaccine containing A/Brisbane/59/2007-like H1N1 virus. Each of the antisera were tested against 10 susceptible (NA-275H) and 21 resistant (NA-275Y) strains of A/Brisbane/59/2007-like H1N1 virus, and the average HI titer for each virus group (susceptible and resistant) calculated for each antisera. The proportion of the antisera samples which inhibited hemagglutination at each progressive antisera dilution based on the average HI titer calculated above was then plotted for the susceptible and resistant virus groups. Error bars represent standard deviation between different virus isolates. (B) HI titers associated with 176 H1 positive antisera identified from 1192 individuals, who had no history of receiving influenza vaccine, were estimated using the A/HK/04633/2008 (NA-275H) and A/HK/17599/2009 (NA-275Y) H1N1 viruses, and plotted as in (A). Statistical significance was determined using the t-test for sera from vaccinated individuals (A) or McNemar test for sera from non-vaccinated individuals (B). Asterisk indicates that the difference in HI reactivity between oseltamivir susceptible and resistant strains is statistically significant (* $p < 0.05$, ** $p < 0.01$).

antibodies present in the general population react differently to the resistant variant, we examined 176 antisera reactive to the A/Brisbane/59/2007 H1N1 virus which had been identified in 2009 from among 1192 individuals who did not have an influenza vaccination history. Consistent with the result obtained with sera from vaccinated individuals, the resistant strains analyzed exhibited lower reactivity towards these 176 sera than the oseltamivir susceptible strains (Fig. 1B). A similar result was observed when a panel of H1 specific monoclonal antibodies was used in the HI test, with resistant virus strains exhibiting little or no reactivity with some of the H1 monoclonal antibodies (Table 1). To confirm whether the above HI assay observations correlate with the ability of antibodies to inhibit virus attachment during infection, the

Table 1
Hemagglutination inhibition test of H1N1 virus with H1-reactive monoclonal antibodies.^a

Seasonal H1N1 viruses	NA ^b (275)	Monoclonal antibodies ^c											
		1D1	2E2	3A8	4C10	5H4	9D5	9E10	10G11	11A1	11E1	15G1	16C1
HK/01236/2008	H	1600	100	400	1600	400	200	400	200	200	100	800	< ^d
HK/02181/2008		12800	100	1600	12800	12800	6400	200	6400	6400	3200	3200	3200
HK/04427/2008		12800	100	400	12800	800	800	<	200	800	200	800	1600
HK/01045/2008		12800	100	200	12800	1600	3200	100	200	800	100	800	1600
HK/03807/2008		12800	200	400	12800	12800	3200	200	3200	3200	3200	3200	1600
HK/04633/2008		12800	100	800	12800	12800	6400	200	1600	1600	1600	1600	1600
HK/03138/2008		12800	200	1600	12800	12800	6400	400	6400	3200	3200	3200	640
HK/02022/2008		12800	200	6400	12800	12800	200	<	800	1600	<	<	3200
HK/17566/2009	Y	12800	<	<	12800		3200	<	<	6400	400	800	320
HK/16418/2009		12800	100	<	1600	<	6400	200	<	3200	<	1600	640
HK/15273/2009		12800	<	200	<	<	6400	<	<	1600	<	<	320
HK/15411/2009		12800	<	<	1600	<	3200	200	<	12800	<	1600	640
HK/17270/2009		1600	<	<	<	<	<	<	<	800	<	<	160
HK/16176/2009		12800	<	<	200	<	100	<	<	3200	<	200	320
HK/62768/2008		12800	<	<	12800	<	3200	<	<	<	<	100	320
HK/65446/2008		12800	<	<	3200	<	1600	<	<	1600	<	<	160
HK/63799/2008		12800	<	<	12800	<	400	<	<	400	<	<	160

^a H1-reactive monoclonal antibodies were raised against the H1N1 virus strain A/Brisbane/59/2007.

^b NA-275H (oseltamivir susceptible), NA-275Y (oseltamivir resistant).

^c HI titer was determined using an antibody dilution range of 1:100 to 1:12800.

^d < Denotes titer under 100.

Table 2
Neutralization of H1N1 clinical isolates with flu vaccine immunized human sera and H1-reactive monoclonal antibodies.

Seasonal H1N1 viruses ^b	Sera/antibodies	NA-275H		NA-275Y	
		HK/04633/2008	HK/03138/2008	HK/17566/2009	HK/16418/2009
Immunized human sera ^a	HS1	320	640	80	80
	HS2	1280	1280	40	<
	HS3	160	160	80	40
	HS4	640	640	160	80
	HS5	<	<	<	<
	HS6	160	640	<	40
	HS7	80	80	<	<
	HS8	160	640	<	<
	HS9	640	640	40	80
	HS10	80	80	40	40
	HS11	320	320	<	40
	HS12	80	80	40	40
	HS13	80	80	160	<
	HS14	<	40	40	40
	HS15	320	320	160	320
	HS16	40	40	160	160
	HS17	640	1280	1280	1280
	HS18	80	80	80	80
	HS19	80	320	40	80
	HS20	640	1280	320	160
H1 monoclonal antibodies	1D1	12800	12800	12800	12800
	2E2	200	400	100	<
	3A8	200	800	<	<
	4C10	12800	12800	6400	1600
	5H4	400	12800	<	<
	9D5	6400	6400	400	3200
	9E10	200	100	<	<
	10G11	800	3200	<	<
	11A1	3200	3200	800	1600
	11E1	1600	6400	<	<
	15G1	800	800	100	200
	16C1	6400	6400	<	6400

Note: Numbers represent highest dilution of antiserum (dilution range 1:10 to 1:1280) or monoclonal antibody (dilution range 1:100 to 1:12800) which still exhibits neutralization activity.

^a HS indicates human sera from immunized individuals.

^b < Denotes titer under 40 for human sera, or under 100 for monoclonal antibodies.

neutralization activity of antisera obtained from vaccinated people and H1 specific monoclonal antibodies against two resistant and two susceptible H1N1 virus strains was compared in a cell-based neutralization assay. As shown in Table 2, the susceptible strains

are inhibited at higher antisera or monoclonal antibody dilutions than the resistant strains. In summary, we found that antibodies against H1N1 virus exhibit less reactivity towards the 275Y resistant variant of H1N1 virus than to wild type virus.

3.2. Phylogenetic and sequence analyses of oseltamivir resistant and susceptible strains

To examine whether oseltamivir resistant and susceptible strains are genetically similar, sequences of HA and NA genes from isolates tested in this study were analyzed. The HA1 and NA phylogenetic trees showed that both oseltamivir resistant and susceptible strains, including the oseltamivir sensitive A/Brisbane/59/

2007 vaccine strain, belong to clade 2B (Fig. 2). This result excludes the possibility that differences in reactivity to antibodies observed among oseltamivir resistant isolates may be caused by genetically distinct variants. Sequence comparison of NA genes from 275H and 275Y H1N1 strains found that there is a D354G substitution in all resistant strains (Supplementary Table 1). Variations in other NA residues were not common. A substitution at D151 (D151E/N/A) of the NA gene has been found to affect the receptor binding

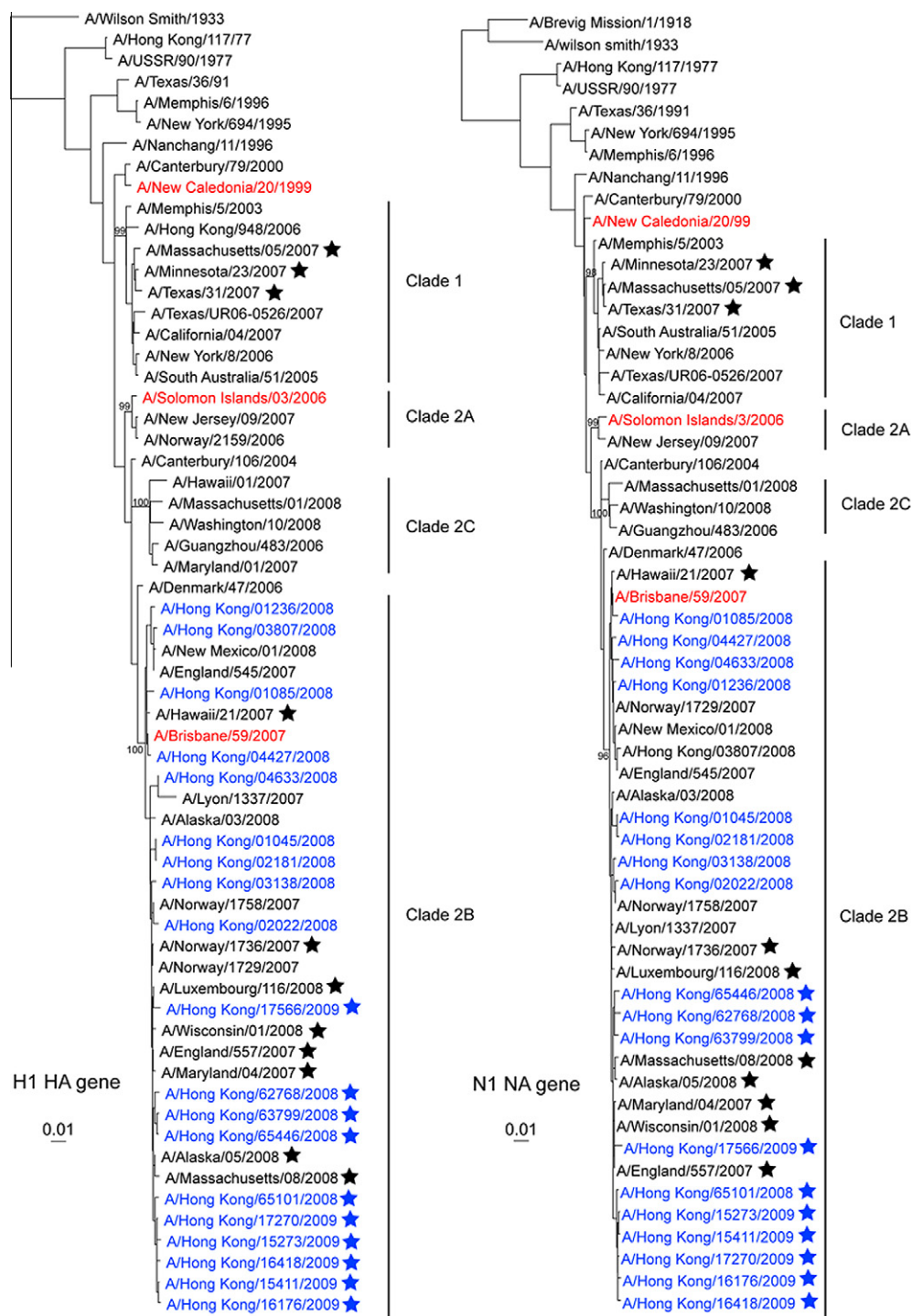


Fig. 2. Phylogenetic trees for the HA (A) and NA (B) genes of H1N1 viruses isolated in Hong Kong during the 2008–2009 influenza seasons. The nucleotide sequences were analyzed with PAUP* software, using the neighbor-joining method with 1000 bootstraps. Sequences of H1N1 viruses characterized in this study are colored blue. Red-hued sequences represent H1N1 influenza vaccine strains recommended by the World Health Organization. Star (*) indicates NA H275Y variant of the H1N1 virus. Genetic lineages of clade 1, 2A, 2B and 2C H1N1 viruses were based on previous reports (Collins et al., 2009; Hauge et al., 2009).

Table 3Hemagglutination inhibition assay of the 2009 pandemic H1N1 virus with monoclonal antibodies.^a

2009 Pandemic H1N1 viruses ^d	NA (275) ^c	Monoclonal antibodies ^b															
		1E12	2B3	2F2	3G3	4A7	4A9	4G12	6B8	6B9	8H9	10B4	14D2	14H2	15H9	18A9	J19B8
California/04/2009	H	<	12800	12800	12800	6400	12800	12800	12800	12800	12800	12800	<	1600	12800	12800	12800
HK/415742/2009		12800	12800	12800	12800	3200	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/419301/2009		12800	12800	12800	12800	1600	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/420518/2009		12800	12800	12800	12800	3200	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/433397/2009		12800	12800	12800	12800	3200	12800	12800	12800	12800	12800	12800	12800	3200	12800	12800	12800
HK/51542/2010		12800	12800	12800	12800	6400	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/45687/2010		12800	12800	12800	12800	3200	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/40835/2010		12800	12800	12800	12800	1600	12800	12800	12800	12800	12800	12800	12800	1600	12800	12800	12800
HK/00783/2010		12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800
HK/02645/2010		12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800
HK/423432/2009	Y	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	12800	3200	12800	12800	12800
HK/23369/2009		12800	12800	12800	12800	3200	12800	12800	12800	12800	12800	12800	12800	3200	12800	12800	12800
HK/FFD/2009		12800	12800	12800	800	6400	12800	3200	12800	12800	12800	12800	12800	1600	12800	12800	12800

^a HI titer was estimated using monoclonal antibody dilutions ranging from 1:100 to 1:12800.^b Monoclonal antibodies used in this study were raised against the 2009 pandemic H1N1 virus strains A/California/04/2009 and A/California/07/2009.^c Ten strains of the 2009 pandemic H1N1 virus characterized between 2009 and 2010 which contain NA-275H and three strains which contain NA-275Y were examined in this study.^d Accession numbers: CY087301, CY087303, CY087292, CY087294, CY087296, CY087298, CY087288, CY087290, CY087286, CY087305, CY087307.

characteristics of human H3N2 viruses, resulting in altered agglutination of red blood cells (Lin et al., 2010). Only one oseltamivir susceptible isolate, A/Hong Kong/02181/2008, was found to contain a D151E substitution. In the HA gene, all resistant strains contain an A189T substitution that is not seen in any of the susceptible strains (Supplementary Table 2). Variations at HA residues 141, 185 and 186 were also found among some resistant variants, but not in susceptible strains. These results indicate that although oseltamivir resistant and susceptible strains are phylogenetically similar, variations occur in both the NA and HA genes of these viruses. The impact of NA D354G, HA A189T and other variations detected in the HA gene on viral fitness and antigenic properties remains to be investigated.

The swine origin pandemic H1N1 virus has replaced previous seasonal H1N1 virus since 2009 and sporadic oseltamivir resistant variants have also been detected in many countries (Chen et al., 2009; Hurt et al., 2011; Meijer et al., 2011; Ujike et al., 2011). To test if resistant and susceptible strains of the pandemic 2009 virus may also exhibit differences, we compared the antigenic reactivity of a panel of monoclonal antibodies specific for the 2009 H1N1 virus towards three resistant and 10 susceptible strains of the 2009 pandemic H1N1 virus, collected during the 2009 and 2010 seasons. In contrast to the result obtained with 2008–2009 seasonal H1N1 viruses, no apparent difference was observed in the reactivity of the panel of monoclonal antibodies with resistant and susceptible strains of the pandemic 2009 virus (Table 3).

3.3. H275Y variants exhibit differential neuraminidase activity

Previous studies showed that H275Y substitution in the NA gene caused changes in the enzymatic properties of the neuraminidase proteins of H1N1 and H5N1 viruses (Collins et al., 2009, 2008; Rameix-Welti et al., 2008). We analyzed the K_M and V_{max} values of some of the isolates used in this study. The three resistant strains analyzed exhibited higher K_M values than susceptible viruses (Table 4), indicating a reduced affinity for substrate. To investigate if the differences in antibody reactivity observed in this study may be due to specific properties associated with variations in the neuraminidase proteins of the tested viruses, we used the neuraminidase inhibitor, zanamivir, to inhibit the neuraminidase activity of both resistant and susceptible strains of H1N1 virus prior to their use in the HI test. Zanamivir binds to a different site on the NA protein than oseltamivir and its action is unaffected by the H275Y substitution in the NA protein of H1N1 virus (Collins

Table 4Enzymatic properties of the neuraminidases of 275H and 275Y H1N1 viruses.^a

Virus strain ^b	NA (275)	K_M (μ M)	V_{max} (OD)
HK/02022/2008	H	16.99	0.421
HK/03138/2008		40.66	0.394
HK/04633/2008		20.68	0.472
HK/16418/2009	Y	68.19	0.243
HK/17270/2009		61.96	1.843
HK/17566/2009		59.05	0.719

^a Enzymatic properties of neuraminidases, indicated by K_M and V_{max} values, were estimated as described in the Section 2.^b Three H1N1 strains of each NA genotype (275H or 275Y) were analyzed in this study.

et al., 2009; Hurt et al., 2009a; McKimm-Breschkin, 2000). To verify the effect of neuraminidase activity on the hemagglutination inhibition assay, the percentage of sera samples ($N = 20$) with HI titer $>1:40$ was estimated in the presence or absence of zanamivir. In contrast to the results obtained when zanamivir was not used, no significant difference in antisera reactivity towards resistant and susceptible strains of H1N1 virus was observed when neuraminidase activity was inhibited (Fig. 3). To further characterize the effect of the H275Y substitution on reactivity between

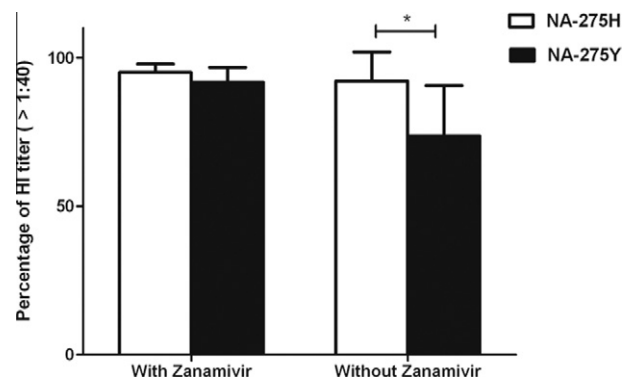


Fig. 3. Testing of the effect of neuraminidase inhibitor treatment on the reactivity of antibodies with oseltamivir susceptible and resistant H1N1 viruses. Hemagglutination inhibition testing was conducted with 20 immunized human sera, with or without pretreatment of viruses with 5 μ M of the neuraminidase inhibitor, zanamivir. Percentage of sera samples with HI titer $>1:40$ represents average from three independent experiments. Error bars represent standard deviations between different isolates. Statistical significance was determined using the McNemar test. Asterisk (*) indicates the difference is statistically significant (p value <0.01).

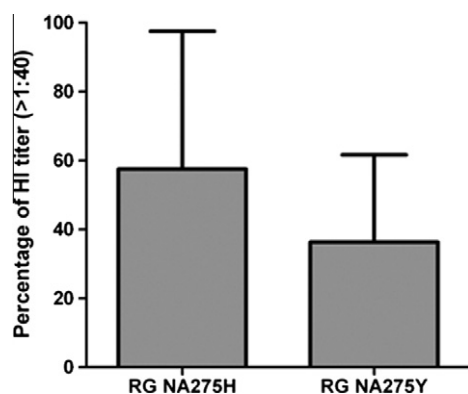


Fig. 4. Comparison of hemagglutination–inhibition activity of antisera against NA 275Y and 275H H1N1 recombinant viruses. HA and NA derived from A/Hong Kong/62768/2008, together with six internal genes from the PR8 strain, were used to construct recombinant viruses. One version contains wild type HA and NA (275Y) genes while the other contains wild type HA and NA with residue 275Y mutated to 275H. Recombinant viruses were tested against 20 sera obtained from seasonal flu vaccine immunized individuals, as described in Fig. 1A. The experiment was repeated three times and data statistically analyzed using the McNemar test. Error bars represent standard deviation.

antibodies and resistant strains, we constructed a pair of recombinant viruses with HA and NA either directly derived from an oseltamivir resistant seasonal H1N1 virus, A/Hong Kong/62768/2008, or with the same HA but the NA of the resistant strain reverse-mutated from 275Y to 275H. The recombinant viruses were rescued in the context of PR8 virus internal genes and the genetic stability of recombinant viruses was verified by sequencing analysis following virus passage in MDCK cells. Using this pair of viruses that differed only at the 275 residue, we repeated the HI assay. Analysis with 20 immunized sera in the HI assay showed that the 275Y virus exhibits statistically lower reactivity compared to the 275H virus (Fig. 4). These results suggest that changes in the NA molecule associated with the H275Y substitution may contribute to the lower antibody reactivity of the 275Y variant.

3.4. Oseltamivir resistant seasonal H1N1 virus is less immunogenic in infected mice than a susceptible strain

To further examine if the altered antigenic properties of the oseltamivir-resistant seasonal H1N1 virus may result in it being less immunogenic *in vivo*, we inoculated mice with equivalent doses of either a oseltamivir resistant clinical isolate of the seasonal H1N1 virus (275Y, A/Hong Kong/62768/2008) or a susceptible strain (275H, A/Brisbane/59/2007) through the nasal route. Serum samples were collected at 3 weeks post-infection and the

levels of antibodies to inoculated virus were tested. Four of the seven mice infected with NA 275Y virus showed 4-fold lower antibody titers in the HI assay than the median level for mice infected with the NA 275H isolate (Table 5). In contrast to the results using clinical isolates, when mice were infected with reverse genetically modified H1N1 viruses where the only difference between oseltamivir resistant and susceptible strains was the H275Y substitution in the NA gene, there was no apparent difference in antibody levels between the 275Y and 275H strains, suggesting that H275Y alone is not sufficient to alter the antibody response in mice (Table 5). Notably, antibody titers from mice inoculated with oseltamivir resistant (A/Hong Kong/423432/2009) are higher than those elicited by susceptible (A/California/07/2009) strains of the 2009 pandemic H1N1 virus (Table 5), further suggesting that the oseltamivir resistant 2009 pandemic H1N1 virus does not possess similar antigenic properties to the 2008–2009 H1N1 NA-H275Y virus. Further analyses of the cross reactivity of antisera found an 8-fold decrease in median HI titer when oseltamivir-resistant isolates were tested against mouse antisera raised against the H1N1 oseltamivir-susceptible strain, A/Brisbane/59/2007 (Table 6). However, no such difference was observed with oseltamivir resistant 2009 pandemic H1N1 isolates tested against antisera raised against the oseltamivir susceptible strain, A/California/07/2009. These results support the notion that the oseltamivir resistant variant of the 2008–2009 H1N1 virus, but not that of the 2009 pandemic H1N1 virus, is less reactive to antibodies induced by the susceptible strain, and may also be less immunogenic, inducing a comparatively weaker antibody response *in vivo*.

4. Discussion

Influenza A virus replication fitness is partially achieved by continuous adjustment of the functional balance between affinity of attachment to receptors by hemagglutinin (HA) and activity of cleavage from cellular receptors by neuraminidase during the virus infection cycle (Wagner et al., 2002). Both HA and NA are primary targets for the host immune response. Mutations at sites or residues associated with functions of the HA or NA proteins of influenza A viruses will lead to an alteration in the balance between the attachment and cleavage processes during virus infection and a re-balance between HA and NA functions will be necessary to regain fitness. The rapidly-gained prevalence of a naturally occurring NA 275Y mutant of H1N1 virus raised concern that a similar trend may occur for other influenza viruses, in particular the 2009 H1N1 virus (Moscona, 2009). It is therefore important to understand the mechanisms underlying the emergence and prevalence of H1N1 influenza viruses carrying oseltamivir resistance mutations.

The rapid rise to prevalence of the naturally occurring H275Y resistant of the H1N1 virus since its emergence in late 2007

Table 5
Hemagglutination inhibition test of H1N1 virus strains with homologous mouse antisera.^a

Inoculated strains	Mouse antisera								Mouse control sera ^b	
	1	2	3	4	5	6	7	Median	1	2
Brisbane/59/2007	160 ^c	80	160	160	160	160	160	160	< ^d	<
HK/62768/2008	40	80	80	80	40	40	40	40	<	<
California/7/2009	320	160	320	320	640	320	320	320	<	<
HK/423432/2009	640	1280	1280	1280	640	1280	640	1280	<	<
RG 62768 NA-275H	320	160	80	160	160	80	160	160	<	<
RG 62768 NA-275Y	160	320	160	160	160	80	-	160	<	<

^a Groups of six or seven mice were inoculated with H1N1 virus through the nasal route and antisera were collected at 3 weeks post-infection. One of the mice inoculated with strain RG 62768 NA-275Y died during the experiment.

^b Mouse control sera were made by inoculated mice with PBS.

^c Numbers represent highest dilution of antiserum (dilution range 1:10 to 1:1280).

^d < Denotes HI titer under 10.

Table 6

Hemagglutination inhibition test of oseltamivir resistant strain reactivity to mouse antisera raised against vaccine strains.

		Serum sample number	Inoculated strain ^a	Resistant viruses		
				Brisbane/59/2007	HK/62768/2008	HK/65101/2008
Seasonal H1N1	1	160 ^b	80	80	40	
	2	80	10	< ^c	<	
	3	160	40	20	20	
	4	160	20	40	20	
	5	160	80	40	80	
	6	160	10	10	<	
	7	160	10	20	10	
	Median	160	20	20	20	
		California/07/2009	HK/FFD/2009	HK/23369/2009	HK/423432/2009	
Pandemic H1N1	1	320	320	320	640	
	2	160	160	160	640	
	3	320	320	160	640	
	4	320	320	320	640	
	5	640	320	640	640	
	6	320	320	320	640	
	7	320	320	320	640	
	Median	320	320	320	640	

^a Both Brisbane/59/2007 and California/07/2009 are vaccine strains.^b Numbers represent highest dilution of antiserum (dilution range 1:10 to 1:1280).^c < Denotes HI titer under 10.

suggested a possible adaptation mechanism involving restoration of fitness and/or gain of an epidemic advantage. Sequence and structural analyses found that the H275Y resistant strains are predominantly clade 2B lineage H1N1 viruses which carry an additional NA substitution, D344N (Collins et al., 2009; Rameix-Welti et al., 2008). Consistent with this observation, the D344N substitution is present in all of the oseltamivir resistant isolates included in this study (Supplementary Table 1). Another study found that permissive mutations at V234M and R222Q, outside of the active enzyme site, significantly increase the amount of NA protein that reaches the cell surface, while the activity per enzyme molecule is either unaffected or slightly decreased (Bloom et al., 2010). Since both the oseltamivir resistant and susceptible H1N1 viruses examined contained these two mutations (Supplementary Table 1), it seems unlikely that these substitutions are associated with the altered antigenic and immunogenic properties of the H1N1 viruses described in this study. It is notable that all oseltamivir resistant strains of seasonal H1N1 virus examined in this study also contain a D354G substitution in the NA gene that is not seen in any of the susceptible isolates (Supplementary Table 1). The D354G substitution is also observed in some other clade 2B H1N1 viruses, however, there is no known structural basis to support this mutation having a major impact on the enzymatic characteristics of NA (Collins et al., 2009; Rameix-Welti et al., 2008; Russell et al., 2006). Examination of the HA gene also revealed exclusive association of the A189T substitution with oseltamivir resistant (H275Y) 2008–2009 H1N1 viruses, as well as variations at positions 141, 185 and 186 in some resistant variants. It remains to be investigated whether these H275Y-coupled mutations in the NA protein provide the capacity to re-balance HA and NA functions, restoring the H275Y mutation-associated defects in protein folding, substrate interaction, and transport of NA, and possibly reinstating fitness of the virus.

For an emerging influenza virus strain to become prevalent, it requires fitness in receptor binding (via HA) and cleavage (via NA), replication efficiency and transmissibility. It is also necessary for a prevalent strain to adapt to or evade host immune surveillance (Palese and Wang, 2011). We found that antisera obtained from vaccine immunized or naturally infected people, and monoclonal antibodies raised against HA of vaccine strains reacted less strongly with H275Y resistant strains of seasonal 2008–2009 H1N1 viruses. Our data also showed that the oseltamivir resistant

strain is less immunogenic in mice. While permissive mutations can restore the optimal functioning of neuraminidases carrying the 275Y mutation, the distinct antigenic and immunogenic properties possessed by the 2008–2009 H1N1 oseltamivir resistant variant may provide a further advantage, allowing the virus to evade the immune system and tolerate neutralizing antibodies. Our data using recombinant viruses varying only by the H275Y substitution suggests that molecular changes caused by H275Y substitution in the NA of the 2008–2009 oseltamivir resistant H1N1 virus can cause reduced reactivity to antibodies raised against the susceptible H1N1 strain in the *in vitro* HI assay (Fig. 4 and Table 6). However, in contrast to the 275Y clinical isolate, which was less immunogenic than the tested 275H isolate, recombinant viruses carrying either NA-275Y or altered from NA-275Y to NA-275H by site mutagenesis show no difference in antibody induction *in vivo* (Table 5). It appears the H275Y substitution alone is insufficient to account for the altered immunogenic properties of 2008–2009 oseltamivir resistant H1N1 viruses. Further study is required to understand the composite roles of substitutions in both HA and NA in conferring replication fitness and the distinct immunogenic properties associated with the oseltamivir resistant H1N1 variant that enabled it to rise to, and sustain, worldwide prevalence between 2007 and 2009.

While resistant variants of the 2009 H1N1 virus carrying the H275Y substitution have been detected (Chen et al., 2009; Hurt et al., 2011; Meijer et al., 2011; Ujike et al., 2011), current circulating strains remain mostly susceptible to oseltamivir. Assessment of the fitness of resistant strains in animal models suggests that the H275Y variant of pandemic 2009 H1N1 virus may not be less transmissible or virulent than wild type (Hamelin et al., 2010; Kiso et al., 2010; Seibert et al., 2010). Our analysis showed that, different to the 2008–2009 seasonal H1N1 viruses, both susceptible and resistant strains of the 2009 pandemic virus are similar in their sensitivity to neutralizing antibodies and their immunogenicity in infected mice (Tables 4 and 5). The 2009 pandemic and the preceding 2008–2009 seasonal H1N1 viruses have traversed different evolutionary paths and exhibit distinct antigenic properties. It is likely that the adaptive changes that allow H275Y variation in the NA of the pandemic H1N1 virus may be different to those which occur in the 2008–2009 H1N1 viruses. As the 2009 pandemic H1N1 virus continues to circulate in humans, and neuraminidase inhibitors are used increasingly for treatment of influenza

virus infection (Sugaya, 2011), it is necessary to monitor the antigenic and immunogenic properties which may allow drug resistant strains of circulating influenza viruses to gain an advantage and rise to prevalence.

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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.2011.11.006.

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