COMMUNICATION

Pseudovirus-based neuraminidase inhibition assays reveal potential H5N1 drug-resistant mutations

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ABSTRACT

The use of antiviral drugs such as influenza neuraminidase (NA) inhibitors is a critical strategy to prevent and control flu pandemic, but this strategy faces the challenge of emerging drug-resistant strains. For a highly pathogenic avian influenza (HPAI) H5N1 virus, biosafety restrictions have significantly limited the efforts to monitor its drug responses and mechanisms involved. In this study, a rapid and biosafe assay based on NA pseudovirus was developed to study the resistance of HPAI H5N1 virus to NA inhibitor drugs. The H5N1 NA pseudovirus was comprehensively tested using oseltamivir-sensitive strains and their resistant mutants. Results were consistent with those in previous studies, in which live H5N1 viruses were used. Several oseltamivir-resistant mutations reported in human H1N1 were also identified to cause decreased oseltamivir sensitivity in H5N1 NA by using the H5N1 NA pseudovirus. Thus, H5N1 NA pseudoviruses could be used to monitor HPAI H5N1 drug resistance rapidly and safely.

KEYWORDS influenza virus, H5N1, neuraminidase inhibitor, drug resistance, pseudovirus

INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus has caused frequent epidemics in domestic poultry in South Asia since 1996 (Xu et al., 1999), resulting in great economic losses and sporadic transmissions from poultry to humans. Recent investigations on H5N1 transmission in ferrets have suggested the possibility of the emergence of mammal-adapted H5N1 that can be transmitted to humans (Herfst et al., 2012; Imai et

al., 2012). In addition to vaccination as a primary option to control influenza, the use of antiviral drugs contributes significantly to an effective prophylaxis and treatment of influenza infections in flu epidemics and pandemics (Bavagnoli and Maga, 2011). Two classes of anti-influenza drugs, including M2 ion channel blockers and neuraminidase (NA) inhibitors are currently available in clinical practice. However, M2 blockers such as adamantane not only produce significant side effects but also rapidly generate drug resistant strains; thus, these drugs have not been recommended for use (Monto, 2003; Hayden, 2006). By contrast, NA inhibitors, including oseltamivir and zanamivir, have been used as primary flu-treatment drugs since 1999 and many countries have procured supplies to prepare for flu or H5N1 pandemics (Peiris et al., 2007; Nguyen et al., 2012). However, NA may undergo mutations, thereby preventing these NA inhibitors to function effectively. For example, H5N1 mutants have become resistant to oseltamivir because of a single NA mutation in H274Y or N294S (N2 numbering) (Le et al., 2005; Yen et al., 2007). Therefore, drug-resistant mutants should be monitored to use anti-flu drugs properly and effectively.

Two approaches, including gene sequencing and functional assay of viral NA activity are commonly used to screen drug resistance to NA inhibitors. Functional assays of viral NA activity can be used to identify a novel drug-resistant genotype. However, functional assays can be limited to HPAI H5N1 surveillance because this virus should only be handled in biosafety level 3 (BSL-3) facilities. Thus, the development of an alternative, safe, and accurate method to study H5N1 drug resistance is important. Pseudotyped viral particles or pseudoviruses have been used as safe surrogates to mimic the structure and surface of many viruses (Garcia and Lai, 2011). Influenza pseudoviruses containing influenza virus surface hemagglutinin (HA) and NA proteins have been used to study NA drug

resistance (Su et al., 2008; Tisoncik et al., 2011). However, this method always fails to generate pseudoviruses because of the mismatch between HA and NA (Zhang et al., 2010). In this study, a simple and effective NA-based pseudovirus was generated to analyze the drug resistance of HPAI H5N1 to NA inhibitors rapidly and safely. This NA-based pseudovirus was investigated using a series of reported H5N1 oseltamivir-resistant mutants and demonstrated that this pseudovirus could identify potential H5N1 drug-resistant mutations.

RESULTS

Suitability of H5N1 NA pseudoviruses for drug-resistance assays

Viral strains exhibit resistance to NA inhibitors mainly because of amino acid mutations in conserved sites around an NA substrate-binding pocket (Ferraris and Lina, 2008). These mutations in conserved residues likely impair NA activity, thereby preventing HA-NA pseudoviruses to be generated (Zhang et al., 2010). This process requires a balance between binding of HA to a sialic acid receptor and NA hydrolysis of this receptor (Wagner et al., 2002). Therefore, we designed a simple NA pseudovirus to analyze the drug resistance of H5N1 NA mutations (Fig. 1). In brief, HIV-based lentiviral particles pseudotyped only with NA from HPAI H5N1 virus (H5N1 NA pseudoviruses) were initially generated by co-transfecting 293T cells with packaging plasmids, including pdelta8.2, plasmid pLuc, and NA expression plasmid (see Methods). The H5N1 NA pseudoviruses obtained from the cell culture supernatants were directly used for enzymatic activity and inhibition assays.

Fig. 2A shows that NA was highly expressed in the cell lysates and the pseudoviruses (see Methods), indicating that NA was successfully incorporated in the pseudoviruses. The successful generation of the H5N1 NA pseudoviruses was further confirmed by observing NA and p24, a constitutive expression protein of pseudoviruses, in the harvested pseudoviruses (Fig. 2A). We further investigated NA activity and drug susceptibility of the H5N1 NA pseudoviruses. We observed a strong fluorescence signal (Fig. 2B), indicating a high NA enzymatic activity after the H5N1 NA pseudoviruses were incubated with an NA fluorogenic substrate MUNANA (see Methods). The NA enzymatic activity of the H5N1 NA pseudoviruses was remarkably inhibited in the presence of NA inhibitors, oseltamivir and zanamivir (Fig. 2B). This result indicated that the H5N1 NA pseudoviruses could be used to analyze the effects of mutations on NA enzymatic activity and drug resistance to NA inhibitors. Fig. 2A also shows that the H274Y mutant had a decreased packaging efficiency compared with wild-type NA, and this finding is consistent with that in a decreased presence of H1N1 NA H274Y mutant on a cellular surface (Bloom et al., 2010). Although the packaging efficiency of mutant NA was different from that of the wild-type NA, this difference does not significantly affect the drug resistance assay, which mainly focuses on relative enzymatic activities in the presence of drugs.

Figure 1. Preparation of neuraminidase (NA) pseudoviruses and their application in influenza drug resistance study. 293T cells were co-transfected with the packaging plasmid pdelta8.2, reporter plasmid pLuc, and NA expression plasmid. NA pseudoviruses were harvested from the cell supernatants. The NA pseudoviruses were used for the assays of NA enzymatic activity and drug responses to NA inhibitors.

Comprehensive validation of the ability of the H5N1 NA pseudoviruses to determine the reported H5N1 drug-resistant mutations

Given the significant effects of HPAI H5N1 virus, its drug susceptibility has also been studied. Previous studies reported that the strain of clade 2.3.4 of H5N1 shows a reduced susceptibility to oseltamivir compared with the strain of clade 1 (Le et al., 2008). To determine whether or not their NA pseudoviruses could identify this difference in drug sensitivity, we compared the oseltamivir responses of H5N1 NA pseudoviruses of A/Anhui/2/2005 (AH05; clade 2.3.4) and A/Vietnam/1194/2004 (VN1194; clade 1) by performing an NA inhibition assay. Table 1 shows that the oseltamivir carboxylate concentrations required to inhibit 50% of NA enzymatic activity (IC_{50}) were 3.35 nmol/L and 0.90 nmol/L for AH05 NA pseudovirus and VN1194 NA pseudovirus, respectively. The AH05 NA pseudovirus exhibited a reduced susceptibility to oseltamivir compared with the VN1194 NA pseudovirus, and this finding is consistent with that in previous reports on live viruses (McKimm-Breschkin et al., 2007; Le et al., 2008). Single mutations of I222M, I222L, S246N, H274Y, and N294S in NA have caused reduced oseltamivir sensitivity of H5N1 virus (Table 1). We further tested their effects by using H5N1 NA pseudoviruses. These reported

Figure 2. Functional analysis of H5N1 NA pseudoviruses.

(A) Western blot analysis of NA in 293T cell lysates and NA pseudoviruses. NA was tagged to detect its expression level (see Methods). Antibodies against the tag were used to detect NA expression. The constitutively expressed proteins GAPDH and P24 were used as loading controls for cell lysates and pseudoviruses, respectively. (B) NA enzymatic activity assay in NA pseudoviruses in the absence or presence of NA inhibitors (oseltamivir and zanamivir).

single mutations were introduced to NA of VN1194 to generate mutant NA pseudoviruses. All of these mutant H5N1 NA pseudoviruses were successfully generated in sufficient amounts required for NA inhibition assay. As expected, all of these mutant H5N1 NA pseudoviruses showed decreased sensitivity to oseltamivir (Table 1). We also calculated their Michaelis-Menten (K_m) and inhibition (K_i) constants to determine their binding affinity to the substrate and oseltamivir, respectively (see Methods). All of the mutant VN1194 NA pseudoviruses showed increased K_i but similar K_m compared with the wild-type VN1194 NA pseudovirus, indicating that these mutants exhibited reduced oseltamivir sensitivities because of the de-

creased affinity between NA and oseltamivir (Table 1).

Considering that H5N1 viruses with H274Y NA mutations are resistant to oseltamivir but susceptible to zanamivir (Hurt et al., 2009), we investigated whether or not NA pseudoviruses bearing H274Y mutation also demonstrated different sensitivities to oseltamivir and zanamivir. Fig. 3 and Table 2 show that the mutant VN1194 NA pseudovirus with H274Y substitution was strongly resistant to oseltamivir (mean-fold increase in IC_{50} ~177) but remained sensitive to zanamivir compared with the wild-type VN1194 NA pseudovirus. This result is consistent with that in studies on live viruses (Yen et al., 2007; Hurt et al., 2009). Thus, H5N1 NA pseudoviruses could be used as an alternative surrogate of live viruses to study NA drug-resistant mutations.

Investigation of potential H5N1 drug-resistant mutations by using H5N1 NA pseudoviruses

We investigated whether or not NA mutations, including I222K, I222R, and S246G that cause reduced sensitivity to oseltamivir in H1N1 (Table 1), could also induce such resistance in H5N1 NA backbone. Based on NA of the VN1194 strain, the mutant NA pseudoviruses bearing the mutations I222K, I222R, or S246G were generated. These three mutants showed a slight reduction in oseltamivir sensitivity in the NA enzyme inhibition assay (Table 1). Therefore, we identified these potential oseltamivir-resistant mutations in H5N1 virus by using the NA pseudovirus.

Dual I222R/H274Y mutation clinically occurs in pandemic H1N1 virus, indicating a significantly higher resistance to oseltamivir than the two single mutations, respectively (Legoff et al., 2012). Although this dual mutation has not been reported to occur in H5N1 virus, we investigated the effect of this dual mutation on H5N1 drug resistance by using NA pseudoviruses. This dual mutation was introduced in VN1194 NA pseudovirus. The sensitivities to oseltamivir and zanamivir were determined by comparing with the NA pseudoviruses containing single mutations. As shown in Table 2, the dual mutation exhibited a remarkably high resistance to oseltamivir and an IC_{50} increase of approximately 3200-fold compared with the wild-type NA pseudovirus. This increase was greater than the increase in IC_{50} of the two single mutations [5.2 (I222R) $\times$ 177.7 (H274Y) = 924]. However, this dual mutation was still sensitive to zanamivir ($IC_{50} < 5$ nmol/L), indicating only a slight increase in IC_{50} (3.1-fold increase in IC_{50} compared with the wild type). Therefore, we identified the effect of the dual mutation on the resistance of H5N1 NA pseudovirus to oseltamivir and zanamivir, and this effect is similar to that observed in H1N1 virus (Nguyen et al., 2010; van der Vries et al., 2011). This result further suggested that this dual mutation could be used as an oseltamivir-resistant marker for H5N1 viruses.

DISCUSSION

Efforts have been devoted to the prevention and control of HPAI H5N1 because of its increasing outbreaks in poultry with

Table 1. Susceptibility of the NA pseudovirus to Oseltamivir

NA Pseudovirus	K_m (μmol/L)		K_i (nmol/L)		IC_{50} (nmol/L)		Mutation ^c	Reports ^d	References
	Mean ^a	Fold change	Mean ^a	Fold change	Mean ± SD ^b	Fold change			
Clade2.3									
AH05*	52.98	-	1.04	-	3.35 ± 1.53	-	-	IC_{50} (3.1–4.8 nmol)	Le et al., 2008
Clade1									
VN-WT**	210.9	1.0	0.14	1.0	0.90 ± 0.21	1	-	IC_{50} (0.04–1.1 nmol)	Le et al., 2008
VN-I222M	338.6	1.6	2.03	14.5	6.17 ± 0.76	6.9	1	H1N1(8)	Deng et al., 2011
							2	H5N1(36)	Hurt et al., 2011
VN-I222L	294.4	1.4	0.86	6.1	2.37 ± 0.83	2.6	-	-	-
							4	H5N1 [K150N + I222L + S246N (77); S246N (24)]	Boltz et al., 2010
VN-S246N	385.0	1.8	1.12	8.0	2.60 ± 1.43	2.9	57	H1N1(4–8)	Hurt et al., 2011
							15	H5N1(24)	Boltz et al., 2010
VN-H274Y	113.2	0.5	45.80	327.1	159.90 ± 37.16	177.7	1174	H1N1(221–2597)	Sheu et al., 2008; Gubareva et al., 2010; Nguyen et al., 2010b
							7	H5N1(911–2502)	Le et al., 2005; Hurt et al., 2009; Ilyushina et al., 2010
VN-N294S	412.4	2.0	4.42	31.6	6.73 ± 1.36	7.5	2	H1N1(197–208)	Abed et al., 2006; Boivin et al., 2011
							10	H5N1(12–138)	Le et al., 2005; Earhart et al., 2009; Ilyushina et al., 2010
VN-I222K	275.7	1.3	3.30	23.6	4.12 ± 0.61	4.6	2	H1N1(28–45)	Nguyen et al., 2010a
							1 ^e	-	-
VN-I222R	201.3	1.0	2.72	19.4	4.64 ± 1.02	5.2	6	H1N1(28–45)	van der Vries et al., 2010; Eshaghi et al., 2011
							-	-	-
VN-S246G	361.1	1.7	1.45	10.4	3.61 ± 2.49	4.0	3	H1N1(5–10)	Sheu et al., 2008
							g ^f	-	-

^a The substrate concentration that resulted in a half-maximal catalytic rate. The enzyme kinetic data were fit to the Michaelis-Menten equation using Graph-Pad Prism5. Values are from two independent experiments.

^b The concentration of NAIs that reduced NA activity by 50% relative to a reaction mixture containing virus but no inhibitor. Values are from three independent experiments.

^c The natural occurring mutations were calculated from 11326 H1N1 sequences and 3015 H5N1 sequences downloaded from NCBI (as of Aug 20, 2012).

^d Drug phenotype is shown according to the referenced studies.

^e A/Thailand/NKFE/2005; GenBank accession No. ABJ98524.

^f GenBank accession No. ACZ05858; ABL08294; ADM95380; ABO30364; ABO30363; ABO30352; ABE97700; ABO20961; AFK10225.

*A/Anhui/2/2005(H5N1); **A/Viet Nam/1194/2004(H5N1); N2 numbering.

sporadic human infections that are associated with high mortality. However, this H5N1 virus is highly infectious and should be handled with extreme caution only in BSL-3 facilities because potential biohazard risks can arise when it spreads outside these facilities (Perez, 2012). Given that the influenza virus continues to mutate, oseltamivir-resistant NA mutations should be monitored to treat H5N1 infections effectively. In this study, a safe and convenient method based on NA pseudoviruses was developed to overcome potential hazards of handling live H5N1 viruses and analyze the resistance of H5N1 virus to NA

inhibitors such as oseltamivir. In this study, we demonstrated that H5N1 NA pseudoviruses exhibited similar sensitivities to live viruses, indicating that NA pseudoviruses could be used to investigate the drug resistance of HPAI H5N1 mutants.

Several oseltamivir-resistant mutations have been identified in seasonal H1N1 viruses and are used as molecular markers to monitor H1N1 drug resistance (Table 1). Among these mutations, I222M, I222L, S246N, H274Y, and N294S were observed and confirmed in live H5N1 viruses. In this study, the H5N1 NA pseudovirus was used to determine whether or not

Figure 3. Assessment of IC_{50} of wild type and mutant H274Y H5N1 NA pseudoviruses to (A) zanamivir and (B) oseltamivir. NA inhibition assay was performed and the dose-response curves were fitted using GraphPad Prism software. IC_{50} s of each NA pseudovirus to NA inhibitors were generated from the fitting curves.

the single mutations I222K, I222R, and S246G with reduced sensitivity to oseltamivir in H1N1 could cause similar effects in H5N1. Our result suggested that these three mutations could be used as molecular markers to monitor H5N1 drug resistance. We also found that the dual mutation I222R/H274Y in H5N1 exhibited similar oseltamivir resistance to that in H1N1.

For H5N1 NA drug-resistance assay, the use of NA pseudoviruses is better than the use of purified NA proteins. H5N1 NA pseudoviruses can be easily prepared without performing NA protein isolation and purification. NA pseudoviruses also show good enzyme stability compared with purified NA proteins that can be easily inactivated because of the lack of membrane environments (Yen et al., 2006; Schmidt et al., 2011). NA enzyme properties can be maintained in the pseudovirus membrane. In this study, > 50% of NA activities of the NA pseudoviruses in the culture medium were retained when stored at 4°C for one week.

H5N1 NA pseudoviruses had evident advantages compared with live H5N1 viruses or purified H5N1 NA proteins in drug-resistance assays on viral sensitivity to NA inhibitor drugs. Thus, the H5N1 NA pseudoviruses that we generated in this study could be used as a surrogate and potential tool to study the drug resistance of HPAI virus and screen antiviral drugs.

MATERIALS AND METHODS

Cell culture

For pseudotyping, 293T human embryo kidney pseudovirus producer cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified essential medium (DMEM; Invitrogen) supplemented with or without 10% fetal bovine serum.

Construction of NA expression plasmids to produce NA pseudovirus

The NA gene was obtained by amplifying the cDNA of the full-length

NA of H5N1 strain, A/Vietnam/1194/2004, or A/Anhui/2/2005 by using a synthetic reverse primer containing a tag (YPYDVPDYA). The tag fused to the C-terminal of NA did not significantly affect the NA enzymatic activity (Fig. S1) as observed in a previous study (Bloom et al., 2010). No difference in oseltamivir sensitivities was found between NA with or without tag (Fig. S2). The tagged NA was cloned in a pVRC expression vector by using Sal I and Not I restriction sites. The other two plasmids used for pseudovirus packaging were pDelta8.2 (Addgene #8455) and pHR'-Luc.

Site-directed mutagenesis

NA mutations were generated by site-directed mutagenesis based on plasmid PCR. In brief, primers with the target residue mutation were designed using the online program Primer X (<http://bioinformatics.org/primerx/>). The plasmid with wild-type NA was used as the template and amplified using high-fidelity Pfu Ultra II Fusion HS DNA polymerase (Agilent) under the following conditions: 94°C for 30 s; followed by 18 cycles of 94°C for 30 s, 50°C for 1 min, 68°C for 10 min; then 68°C for 10 min. The original plasmid template in the PCR reaction mixture was removed by digesting with Dpn I (New England Biolabs) for 3 h at 37°C. The mixture was transformed to *Escherichia coli* Top 10 (Invitrogen) strain and the plasmids were extracted. The presence of the introduced mutation and the absence of additional unwanted mutations were verified by sequencing the whole inserted NA sequence in the plasmids.

NA pseudovirus production

NA pseudoviruses were produced by transfecting 293T cells with three plasmids, including NA expression plasmid, pDelta8.2, and pLuc using PEI (Sigma) by following Kuroda et al.'s procedure (Kuroda et al., 2009). The plasmid pLuc was used because its genetic elements (such as psi-sequence and long terminal repeats) are needed for pseudovirus packaging (Garcia and Lai, 2011). We also found that the plasmid pLuc could enhance the packaging efficiency of NA pseudovirus. At 16 h post-transfection, the supernatants were removed and replaced with DMEM. After another 48 h of transfection, the NA pseudoviruses

Table 2. Susceptibility of NA pseudoviruses to NA inhibitors

NA ^a	K_m ($\mu\text{mol/L}$)		Oseltamivir				Zanamivir			
			K_i (nmol/L)		IC_{50} (nmol/L)		K_i (nmol/L)		IC_{50} (nmol/L)	
	Mean ^b	Fold change	Mean ^b	Fold change	Mean $\pm$ SD ^c	Fold change	Mean ^b	Fold change	Mean $\pm$ SD ^c	Fold change
WT	210.9	1.0	0.14	1.0	0.90 $\pm$ 0.21	1.0	0.51	1.0	1.09 $\pm$ 0.10	1.0
H274Y	113.2	0.5	45.80	327.1	159.9 $\pm$ 37.2	177.7	0.13	0.2	0.59 $\pm$ 0.04	0.5
I222R	201.3	1.0	2.72	19.4	4.64 $\pm$ 1.02	5.2	1.81	3.6	2.37 $\pm$ 0.64	2.2
I222R+H274Y	424.5	1.8	1486	10610	2886 $\pm$ 198	3206	2.00	3.9	3.41 $\pm$ 0.03	3.1

^a The NA gene was derived from A/Viet Nam/1194/2004(H5N1).

^b Values are from two independent experiments.

^c Values are from three independent experiments.

were collected from the supernatants by filtering through a 0.22- μm Durapore PVDF membrane filter (Millipore).

Western blot analysis of NA expression and incorporation in pseudoviruses

The pseudoviruses were first concentrated and purified by ultracentrifugation at 10,000 g at 4°C for 3 h. These pseudoviruses were then resuspended in PBS. The NA expression in 293T producer cells was analyzed after the pseudoviruses were harvested. Cells in six-well plates were washed twice with PBS to remove residual culture medium and then suspended in 500 μL of PBS. Each cell suspension (20 μL) was mixed with 4 $\times$ sample buffer (Invitrogen), heated to 100°C for 5 min, and analyzed by western blot.

For western blot analysis, the prepared cell lysate and pseudovirus samples were subjected to electrophoresis on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen). After electrophoresis, the separated proteins were transferred to PVDF membranes (Pall Corp.) according to a semi-dry transfer method.

For NA detection, the membranes were blocked with skim milk overnight at 4°C and then incubated with a primary antibody against rabbit anti-HA tag polyclonal antibody (Cwbio) for 1 h at room temperature. After three washes in PBS, these membranes were incubated with a horseradish peroxidase-labeled secondary antibody for 1 h at room temperature and washed thrice in PBS. NAs were then visualized using an enhanced chemiluminescence western blotting detection reagent (Amersham). Constitutive expression gene GAPDH and P24 were used as loading controls for cell lysates and pseudoviruses, respectively.

NA enzyme activity and NA inhibition assay

Modified fluorometric assay was performed to determine the NA activity of NA pseudoviruses (Potier et al., 1979). The NA enzyme activities in serial dilutions of each pseudovirus were determined at pH 6.5 with 33 mmol/L 2-(N-Morpholino)ethanesulfonic acid hydrate (MES; Sigma-Aldrich), 4 mmol/L CaCl_2 , and 100 $\mu\text{mol/L}$ fluorogenic substrate [2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA); Sigma-Aldrich]. The reaction was terminated with a stop buffer (0.5 mol/L NaOH, 25% ethyl alcohol) after the mixture was incubated at 37°C for 1 h. The relative fluorescence unit (RFU) of the released 4-methylumbelliferone was determined using a GENios Plus reader (Tecan Inc.) at excitation and emission wavelengths of 360 and 460 nm, respectively.

For NA inhibition assay, the pseudoviruses were diluted to obtain 30,000 RFU to 50,000 RFU to ensure the linearity of the enzymatic reaction over time. Each cell suspension sample of diluted pseudoviruses (50 μL) was initially incubated with 50 μL of NA inhibitors (oseltamivir and zanamivir) at different concentrations from 0.03 nmol/L to 30,000 nmol/L at 37°C for 30 min. The mixtures were then incubated with 50 μL of MUNANA at a final concentration of 100 $\mu\text{mol/L}$ in an assay buffer at 37°C for 1 h. The reaction was terminated by adding 100 μL of stop buffer. IC_{50} was calculated using a nonlinear regression function in GraphPad Prism5. The Michaelis-Menten kinetic parameters (K_m and K_i) of the NA pseudoviruses were calculated according to a previously described method (Barrett et al., 2011).

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