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Characterization of Drug-Resistant Influenza A(H7N9) Variant Viruses Isolated from an Oseltamivir-Treated Patient in Taiwan

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Abstract

Background. Patients contracting influenza A(H7N9) often developed severe disease causing respiratory failure. Neuraminidase (NA) inhibitors (NAIs) are the primary option for treatment, but information on drug-resistance markers for A(H7N9) is limited.

Methods. Four NA variants of A/Taiwan/1/2013 (H7N9) virus containing a single substitution (NA-E119V, NA-I222K, NA-I222R or NA-R292K), recovered from an oseltamivir-treated patient, were tested for NAI susceptibility *in vitro*; their replicative fitness was evaluated in cell culture, mice and ferrets.

Results. NA-R292K led to *highly reduced inhibition* by oseltamivir and peramivir, while NA-E119V, NA-I222K and NA-I222R caused *reduced inhibition* by oseltamivir. Mice infected with any virus showed severe clinical signs with high mortality rates. NA-I222K virus was the most virulent in mice, whereas virus lacking NA change (NA-WT) and NA-R292K virus seemed the least virulent. Sequence analysis suggests that PB2-S714N increased virulence of the NA-I222K virus in mice; NS1-K126R, alone or in combination with PB2-V227M, produced contrasting effects in NA-WT and NA-R292K viruses. In ferrets, all viruses replicated to high titers in the upper respiratory tract, but produced only mild illness. NA-R292K virus, showed reduced replicative fitness in this animal model.

Conclusions. Our data highlight challenges in assessment of replicative fitness of H7N9 NA variants emerged in NAI-treated patients.

Recent human infections in China with an avian influenza A(H7N9) virus resulted in substantial morbidity and mortality [1, 2] and provoked global public health concern. The majority of infected patients, most of whom reported direct contact with poultry or visiting live bird markets, were hospitalized and suffered acute respiratory syndrome [3, 4]. Unlike the highly pathogenic avian influenza (HPAI) A(H5N1) virus, the H7N9 virus lacks molecular markers associated with "high pathogenicity" in chickens and causes mild or no disease in poultry, hindering control efforts [3, 5].

Neuraminidase (NA) inhibitors (NAIs) have been used for treatment of influenza. Two NAIs, oral oseltamivir and inhaled zanamivir, are FDA-approved, while intravenous zanamivir is available for compassionate use in severely ill hospitalized patients in the United States (U.S.) [6]. A long-acting inhaled NAI, laninamivir, is available in Japan [7] and intravenous peramivir is marketed in South Korea, Japan [8] and China [9]. Patients who contracted H7N9 were commonly treated with oseltamivir [3, 10, 11], because the virus is resistant to M2 blockers. The therapeutic effectiveness of NAIs, however, can be compromised by emergence of drug-resistant variants.

Molecular markers for NAI resistance are subtype-specific and not well-characterized for H7N9. It was reported that A/Shanghai/1/2013 (Shanghai/1), collected from the patient soon after initiation of oseltamivir treatment, contained the NA substitution, Arg292Lys (R292K, N2 amino acid numbering; R289K in 2013 H7N9 numbering) [3, 12]. This substitution was associated with *in vitro* resistance to NAIs in the N9 subtype [13] and has been reported in several H7N9 viruses [3, 11, 14, 15], at least one of which was recovered from a deceased patient [11]. Furthermore, a mixture of 119Glu/Val (119E/V) was detected in an H7N9 virus collected from a patient following oseltamivir treatment; the patient died of acute respiratory distress syndrome [16]. Both NA changes, R292K and E119V, were previously associated with oseltamivir resistance in patients infected with seasonal H3N2 viruses [17-19]. While oseltamivir resistance-conferring NA substitutions in H3N2 seasonal influenza viruses were detected predominantly in children and/or immunocompromised patients [17, 19-21], H7N9 NA variants have been recovered from otherwise healthy adults [3, 11, 14, 15]. The efficacy of oseltamivir treatment of patients infected with H7N9 viruses containing NA substitutions, as well as replicative fitness of the emerged NA variants remains unknown.

Substitutions in the NA active site are known to impair enzymatic activity and may affect virus fitness. For example, replication of H3N2 virus containing NA-R292K was impaired when tested in ferrets [22]. However, oseltamivir-resistant viruses can overcome reduction in fitness; seasonal H1N1 viruses carrying NA-H275Y spread globally during the 2008−2009 influenza seasons [23]. Their replicative fitness and transmissibility were comparable to drug-susceptible viruses in mice, guinea pigs and ferrets [24-27]. At present, information on the fitness of drug-resistant H7N9 viruses is limited. The Shanghai/1 NA-R292K virus was shown to replicate efficiently in cell culture [28]. Recombinant H7N9 viruses with or without NA-R292K displayed comparable virulence in mice [29, 30] and transmissibility in guinea pigs [30].

Virus population with a 292R/K mixture was reported in the sample collected from a patient in Taiwan on April 24th, 2013 [14]. The specimen was propagated once in chicken eggs (E1) and shared with the U.S. Centers for Disease Control and Prevention (CDC) for further analysis. In addition to NA-R292K, pyrosequencing identified changes at conserved residues NA-E119V, NA-I222K or NA-I222R in the E1 isolate. The latter two substitutions have not previously been reported in H7N9 viruses, but were shown to reduce oseltamivir inhibition in both N1 and N2 NA subtypes [31-34]. The NAI susceptibility of the four identified NA variants was assessed in an NA inhibition (NI) assay, and their replicative fitness and virulence were determined in mice and ferrets.

METHODS

Detailed information on viruses, sequence analysis, plaque purification, NA inhibition (NI) assay and statistical analysis are in Supplementary Materials.

Ethics statement. Animals were housed and experiments conducted in strict compliance with guidelines of the CDC Institutional Animal Care and Use Committee in accordance with PHS Policy, the Animal Welfare Act (USDA), and the Guide for Animal Care and Use of Laboratory Animals. Animal protocols for working with ferrets and mice were approved by the CDC IACUC committee. All procedures were performed under animal biosafety level 3+ conditions, and animal welfare was observed daily.

Infectivity and virulence of H7N9 viruses in mice. Pathogen-free female BALB/c mice (6−8 weeks old) were used (Jackson Laboratory, ME), and acclimatized for at least 3 days prior to virus inoculation. The MLD_{50}s (n=4/group) and MID_{50}s (n=3/group) were determined by intranasally inoculating animals with 10-fold serial dilutions of virus (10^1−10^5 $TCID_{50}$; 50 µl per mouse). During the course of infection, animals were observed for disease signs and physical abnormalities. For the MID_{50} determination, lungs were collected on day 3 post-inoculation, washed thoroughly in phosphate-buffered saline (PBS) and homogenized (Omni Bead Ruptor 24, OMNI International, GA) in 1 ml of PBS. Cellular debris was cleared by centrifugation at 2000×g for 10 min. Supernatants were serially diluted and used for infection of MDCK-SIAT1 cells to determine infectious virus titers using $TCID_{50}$ assay.

Replicative fitness of H7N9 viruses in ferrets. Male Fitch ferrets (*Mustela putorius furo*) aged 3–5 months old (Triple F Farms, PA), and serologically negative by hemagglutination inhibition (HI) assay for currently circulating A(H1N1)pdm09, A(H3N2) and type B influenza viruses, were used in this study. Ferrets were single-housed and monitored for at least 3 days for acclimation, and to establish baseline body temperature prior to starting the study. Clinical signs of illness, such as activity level, sneezing, nasal and ocular discharge, (details for scoring in Supplementary Materials) and body weights were recorded daily throughout the 14 day study. Body temperature was measured twice daily by subcutaneous implantable temperature transponders (Bio Medic Data Systems Inc., DE). Intranasal inoculations (3−4 ferrets/group) were performed under anesthesia, induced by intramuscular administration of a ketamine/xylazine/atropine mixture (25, 2 and 0.05 mg/kg body weight, respectively), using 10^6 $TCID_{50}$ inoculation dose (0.5 ml total volume; 250 µl per nostril).

Nasal washes were collected daily (under anesthesia) for 10 days post-inoculation by flushing both nostrils with 1 ml of PBS, and further processed for determination of infectious virus titers, inflammatory cell counts and protein concentration. Briefly, nasal washes were centrifuged at 1000xg for 10 min. The cell pellet was resuspended in PBS, and inflammatory cells counted using the Scepter 2.0 handheld automated cell counter (EMD Millipore, MA). The protein concentration in cell-free nasal wash supernatants was determined using the BCA Protein Assay Kit (Thermo Scientific, IL). Serum samples were collected 14 days post-inoculation, treated

with receptor-destroying enzyme at 37°C for 18 hours, heat-inactivated at 56°C for 30 min, and tested by HI assay using the respective virus with 0.5% packed turkey erythrocytes.

RESULTS

Isolation and sequencing of NA variants of A/Taiwan/1/2013 (H7N9) (Taiwan/1)

Multiple swab samples were collected from a patient infected with H7N9 in Taiwan, who received prolonged oseltamivir treatment, starting April 16th, 2013 [10]. The emergence of virus with R292K was detected in samples collected on April 22nd and April 25th [15]; a 292R/K mixture was reported in the sample collected on April 24th [14]. The April 24th specimen was propagated once in chicken eggs (E1) prior to Sanger sequence analysis [Global Initiative on Sharing All Influenza Data (GISAID) Accession No. EPI445909−EPI445916, Table S2] and was shared with the U.S. CDC for further analysis.

Pyrosequencing analysis of the E1 isolate revealed nucleotide polymorphisms at three conserved amino acid residues in the NA enzyme active site: E119, I222, and R292. Since each change could affect NAI susceptibility, plaque purification of the E1 isolate was performed. Over 100 individual plaque-purified viruses were screened using NA gene pyrosequencing and Sanger sequencing. The most abundant plaque-purified NA variant was I222R (49%), followed by R292K (23%), E119V (22%), I222K (4%) and wild type (WT, 1%). In all instances, substitutions occurred at the second nucleotide in the respective triplet: R292K (AGG→AAG), E119V (GAA→GTA), I222R (ATA→AGA), and I222K (ATA→AAA). No analyzed NA variant contained changes at more than one of these substitutions. The four NA variants and WT differed by only a single amino acid in the NA and were used in all subsequent experiments (Table 1).

Full-genome sequences of the plaque-purified viruses were compared to the original Taiwan/1 E1 isolate consensus sequence. Besides the NA changes, all viruses differed from each other and the E1 consensus sequence by at least two additional amino acid substitutions (Table 1). The NA-WT virus differed from the E1 consensus by four amino acid changes: PB2-V227M, PA-V554I, HA-N281S, and NS1-K126R. One substitution, PB2-V227M, was unique to this virus and not found in other H7N9 sequences available in GISAID. The NS1-K126R was rare with a K/R mixture only detected in the NS of NA-R292K, and no other virus. The NA-E119V virus had arginine (R) at PA-556, while the other viruses had the glutamine (Q) consensus sequence at this position. The

NA-I222K virus possessed asparagine (N) in PB2-714; other viruses contained serine (S). The HAs of four plaque-purified viruses differed from the E1 consensus sequence by a single amino acid (N281S), whereas the NA-R292K contained D340G (Table 1). Notably, the HA sequence of all five viruses maintained the presence of proline (P) at HA-226, which was present only in the Taiwan/1 consensus sequence in GISAID.

All plaque-purified viruses contained lysine (K) at PB2-627 and aspartic acid (D) at PB2-701, as seen in the two most studied H7N9 viruses to date, Anhui/1 and Shanghai/1 [3, 35]. The first substitution has been shown to increase virulence of H7N9 [36] and HPAI H5N1 [37] viruses in mice. Sequences of H7N9 viruses isolated directly from poultry or environmental samples contain PB2-627E, while both K and E are found in human isolates. The Taiwan/1 viruses contained M2-S31N, a marker of resistance to M2 blockers found in all 2013-2014 H7N9 viruses.

In vitro susceptibility to NAIs

Susceptibility of plaque-purified viruses to five NAIs (oseltamivir, zanamivir, peramivir, laninamivir, and A-315675) was determined in the NI assay. The NA-R292K virus had the highest IC_{50}s (Table 2) with the >1,000-fold increase in oseltamivir and peramivir IC_{50}s consistent with previous reports [12, 28-30]. The NA-E119V virus had the second highest oseltamivir-IC_{50} (84-fold increase) together with a 9-fold increase in zanamivir-IC_{50}, with no effect on inhibition by peramivir, laninamivir, or A-315675. The NA-I222K and NA-I222R viruses shared a similar resistance profile; oseltamivir-IC_{50}s were elevated by 32−37-fold, while 6−14-fold increases were detected for the remaining four NAIs. Applying the WHO Antiviral Working Group (AVWG) criteria [38], the NA-R292K virus was characterized as exhibiting highly reduced inhibition by oseltamivir and peramivir, and reduced inhibition by zanamivir, laninamivir and A-315675. The NA-E119V virus exhibited reduced inhibition by oseltamivir only. The acquisition of NA-I222R or NA-I222K resulted in reduced inhibition by oseltamivir and laninamivir, while NA-I222R also conferred reduced inhibition by zanamivir.

Virus replication in cell cultures.

Assessment of the replicative potential of the NA variants of the Taiwan/1 virus was conducted in two cell lines, conventional MDCK and MDCK-SIAT1 cells that overexpress α-2,6-NeuAc-containing receptors [39]. Cell

monolayers were inoculated at a low multiplicity of infection (MOI=0.0001) and infectious virus yields were determined at 8, 24, 48, and 72 hours post-infection. Very low or no virus titers were detected at 8 hours post-infection in both cell lines. In MDCK cells, all viruses showed efficient replication and produced comparable titers, which peaked at 24−48 hours post-infection (Figure 1A). In MDCK-SIAT1 cells, NA-WT, NA-E119V, NA-I222K and NA-I222R viruses replicated as efficiently as in MDCK cells, their titers peaking at 24 hours post-infection (Figure 1B). In contrast, the NA-R292K virus exhibited delayed growth with infectious titers 2−3-logs lower than those of the other viruses, with the highest titer at 48 hours post-infection. The NA-R292K virus titer was significantly lower than that for the WT virus at 24 ($p<0.0001$) and 72 ($p=0.0175$) hours post-infection (Figure 1C). Therefore, replication of the NA-R292K virus was notably attenuated in MDCK-SIAT1, but not in MDCK cells.

Infectivity and virulence in mice

To determine whether the NA variants of Taiwan/1 virus were attenuated in a mouse model, BALB/c mice were inoculated with 10-fold serially diluted viruses (10^1−10^5 $TCID_{50}$ per mouse) and their lungs harvested day 3 post-inoculation to detect the presence of infectious virus. The mouse infectious dose 50% (MID_{50}) for the NA-WT virus was 32 $TCID_{50}$. Unexpectedly, less than 10 $TCID_{50}$ were required to infect 50% of animals with the four NA variant viruses, including the NA-R292K virus (Table 1). Infections with 10^4 and 10^5 $TCID_{50}$ of any virus resulted in rapid weight loss (Figure 2) and severe signs of illness, including lethargy, ruffled fur, hunched posture and dyspnea. NA variants with a substitution at I222 caused rapid weight loss and 100% lethality with doses as low as 10^2 and 10^3 $TCID_{50}$ (Figure 2, D and F), while the NA-WT virus produced a similar effect only at the highest challenge dose, 10^5 $TCID_{50}$ (Figure 2J). The NA-E119V virus caused 100% lethality in animals infected with 10^4 $TCID_{50}$ (Figure 2H). Notable differences in virulence were also observed based on comparison of the mouse lethal doses 50% (MLD_{50}) (Table 1). The NA-I222K virus was the most virulent ($10^{1.5}$ $TCID_{50}$), while the NA-WT and NA-R292K viruses exhibited 1,000-fold lower virulence ($10^{4.5}$ $TCID_{50}$). The NA-E119V and NA-I222R viruses showed intermediate virulence.

Replicative fitness in ferrets

For ferret model experiments, animals were inoculated intranasally with 10^6 $TCID_{50}$ of virus, after which nasal washes were collected daily for 10 days to assess infectious virus titers, number of inflammatory cells and protein concentrations. All viruses replicated to comparable titers peaking (~10^6 $TCID_{50}$/ml) between days 2 and 4 post-inoculation (Figure 3A). Notably, replication of the NA-R292K virus was delayed; no virus was detected in two of four animals on day 1 post-inoculation, yet these two ferrets shed virus on day 7 post-inoculation. The NA-R292K virus titers were approximately 1−2 logs lower than the NA-WT virus on days 1, 2 ($p=0.004$) and 3 ($p=0.003$) post-inoculation. Quantitative virus shedding was measured, defined as the area under the curve (AUC) during days 1−6 (days 1−7 for NA-R292K virus), and expressed as $log_{10}TCID_{50}$/ml. The AUCs were 29.3 for NA-WT, 29.0 for NA-E119V, 26.6 for NA-I222K, 25.9 for NA-I222R and 26.6 for NA-R292K viruses, indicating a trend towards lower replication rates of NA variants in the ferret's upper respiratory tract (URT), with the exception of NA-E119V virus.

Inflammatory cell counts (Figure 3B) and total protein concentrations (Figure 3C) in nasal washes peaked days 5 and 7 post-inoculation with all five viruses; both parameters were lower ($p<0.05$) in NA-R292K virus-infected animals during days 5−9 post-inoculation. NA variants maintained their substitutions during the shedding period (Table S1).

Unlike the severe disease observed in mice, all viruses produced only modest clinical symptoms in ferrets. No signs of lethargy were seen in any of the ferret groups and no infected ferrets had remarkable weight loss over the course of infection (Figure 3D). Notably, ferrets infected with the NA-R292K virus did not experience weight loss and gained body weight at a faster rate than those infected with the other H7N9 viruses tested. Body temperatures were stable in all infected animals across the course of infection (Figure 3E). Intermittent pyrexia (≥1.5°C above baseline) was detected in one animal infected with the NA-I222R virus early and late post-inoculation. Sera collected from inoculated ferrets on day 14 post-inoculation showed seroconversion against homologous H7N9 virus, with hemagglutination inhibition (HI) titers ranging from 160 to 1280.

DISCUSSION

The presence of viral quasispecies in the NA genes of viruses recovered from patients undergoing antiviral treatment is not unexpected. Analysis of individual plaques of Taiwan/1 (H7N9) virus led to recovery of NA variants without or with one change at residues E119, I222, or R292. Laboratory data for virus with NA-R292K, the most commonly detected substitution in the H7N9 subtype, indicate clinically relevant resistance to oseltamivir and, possibly, peramivir. Thus, R292K may reduce the effectiveness of treatment and prophylaxis with these drugs.

For risk assessment purposes, it was imperative to investigate the replicative fitness of the identified NA variants. R292 participates in catalysis and interacts directly with the sialic acid-containing receptors, while E119 and I222 are framework residues that support the NA binding pocket [40]. Therefore, NA-R292K is likely to cause a greater functional loss of NA enzymatic activity [28, 41] than the framework substitutions, potentially leading to a greater deficit of virus replicative fitness.

In this study, the NA-R292K virus replicated to similar high titers as the NA-WT virus in MDCK cells, but showed less efficient replication in MDCK-SIAT1 cells modified to over-express α2,6-NeuAc-receptors [39]. Because the need for functional NA is greater when virus replicates *in vivo*, we anticipated reduced replicative fitness and virulence of the NA variants in animals. To prove this hypothesis, we used two animal models, mice and ferrets. When tested in ferrets, all four NA variants and the NA-WT virus replicated efficiently according to high titers in nasal washes which peaked on days 2−4 post-inoculation. However, all viruses caused only mild disease signs in ferrets, despite the use of a high inoculation dose (10^6 $TCID_{50}$ per animal). The NA-R292K virus appeared to be the most attenuated, based on fewer counts of inflammatory cells and lower protein levels in nasal washes and a steady increase in body weights. Notably, nasal wash titers of the NA-R292K virus were significantly lower than those of the NA-WT in the first three days post-inoculation, suggesting impaired replicative fitness. Similarly, Yen et al. [42] demonstrate that NA-R292K virus (Shanghai/1 background) showed competitive fitness loss in ferrets; this virus, however, transmitted at comparable efficiency as WT virus to direct or respiratory droplet contact animals.

Unexpectedly, the NA-WT virus showed ~3-fold reduced infectivity in mice (MID_{50} 32 vs. <10) compared to NA variants. Moreover, NA-WT virus required a much greater dose to produce lethality in mice,

when compared to the three NA variants (E119V, I222R and, especially, I222K). The NA-R292K showed an MLD_{50} similar to that of the NA-WT virus, consistent with the recent report [30] indicating comparable virulence of recombinant Shanghai/1 viruses, with or without NA-R292K. Of note, use of different mouse strains, e.g. BALB/c vs. C57BL/6, infected with the same H7N9 virus resulted in substantial differences in pathogenicity and inflammatory responses [43].

One limitation of the present study was a lack of the original clinical sample to characterize a quasispecies composition and to recover NA variants. Another limitation was using plaque-purified versus reverse-genetically generated viruses, to address the effect of each NA change on virus fitness. Nevertheless, our approach, coupled with full-genome sequence analysis, provided valuable insights into viral phenotypes, which would have not been obtained otherwise. The plaque-purified NA-WT showed reduced infectivity and virulence in mice. This virus contained two substitutions PB2-V227M and NS1-K126R, not seen in any other H7N9 viruses, including the published Taiwan/1 sequence. Peculiarly, the NA-R292K virus possessed a mixture of R/K in NS1-126 and it also exhibited reduced virulence in mice. Although the role of NS1-K126R in virulence of H7N9 viruses has not been demonstrated, introduction of NS1-K126R in the A/Puerto Rico/8/34 (H1N1) virus led to increased viral yields in cell culture and virulence in mice [44]. In contrast, this substitution was shown to cause a 10-fold decreased replication of a recombinant H3N2 virus in cell culture [45]. In our study, NS1-K126R had no apparent effect on the H7N9 virus replication in ferrets, indicating its host-specific nature.

The NA-I222K virus showed the highest virulence in mice and contained a substitution at PB2-S714N, not found among H7N9 sequences in GISAID. A switch from serine to arginine at PB2-714 was demonstrated to increase viral RNA transcription activities *in vitro* [46]. The potential role of asparagine at PB2-714 remains unknown. The identified HA substitutions (HA-S281N and HA-D340G) are located in positions not known to affect receptor-binding or antigenicity; the role of other identified internal gene mutations is also unknown at this time.

Overall, interpretation of results used for risk assessment is challenging. The NA variants were highly virulent in mice, but the results were complicated by the presence of other substitutions in the viral internal genes. In the ferret model, animals were asymptomatic when infected with any H7N9 virus, which is contrary to what

was observed in humans. Because Taiwan/1 virus caused severe disease in the patient, these findings in ferrets should be interpreted cautiously.

In summary, we report recovery of four NA variants from the Taiwan/1 H7N9 virus collected from an oseltamivir-treated patient. The lack of an apparent compromise in the replicative fitness of these NA variants is concerning. Close monitoring of viruses for changes at these NA residues needs to be conducted, particularly in patients treated with NAIs. Ideally, specimens should be collected systematically from patients infected with H7N9 virus, regardless of illness severity or treatment, to better understand the emergence of NA variants and quasispecies dynamics. This study also highlights the need for development of novel anti-influenza drugs and drug combination therapies.

Notes

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Figure legends

Figure 1. Replication kinetics of H7N9 virus isolated from a patient in Taiwan. MDCK (A) or MDCK-SIAT1 (B) cells were infected with either plaque-purified virus at an MOI=0.0001. Virus-containing supernatants were collected at the indicated time points, and virus titers were determined in MDCK-SIAT1 cells. The statistically significant difference (*) between wild type and R292K viruses are shown (C). Dotted lines indicate limit of virus titer detection.

Figure 2. Virulence of Taiwan/1 (H7N9) NA variants in mice. Animals (n=4/group) were intranasally inoculated with 10^1, 10^2, 10^3, 10^4 or 10^5 $TCID_{50}$ of indicated viruses. Body weight change (A, C, E, G, I) and survival (B, D, F, H, J) were monitored daily. Mice that lost ≥ 25% (dotted line) of their baseline weight were humanely euthanized. Each value (A, C, E, G, I) represents the average percent (%) of body weight loss ± SD.

Figure 3. Replicative fitness of Taiwan/1 (H7N9) NA variants in ferrets. Animals (n=3−4/group) were intranasally inoculated with 10^6 $TCID_{50}$ of the indicated viruses. Nasal washes were collected daily for 10 days, and virus titers (A), inflammatory cell counts (B) and protein concentration (C) were determined. The body weight change (D) and temperature (E) were recorded daily. The statistically significant difference (*) in nasal wash virus titers between wild type and R292K viruses is shown. Dotted lines indicate limit of virus titer detection. SD and (*) are not shown if less than three animals shed virus.

Tables

Table 1. Comparison of amino acid sequences of plaque-purified NA variants of A/Taiwan/1/2013 (H7N9) virus and mammalian pathotypes.

A/Taiwan/1/2013 (H7N9)	Viral proteins with amino acid differences[e]												Number of amino acid substitutions	MID_{50}[c]	MLD_{50}[c] (fold increase)	Compared to NA-WT virus	
	PB2		PB1	PA			HA[a]		NA[b]			NS1				Virulence in mice	Replicative fitness in ferrets
	227	714	632	90	554	556	281	340	119 (115)	222 (219)	292 (289)	126					
E1 isolate[d]	V	S	A	V	V	Q	N	D	E	I	R	K	4	–	–	–	–
NA-WT	M				I		S					R	–	32	$10^{4.5}$	–	–
NA-E119V						R	S		**V**				5	<10	$10^{2.7}$ (68)	Increased	Comparable
NA-I222K		N			I		S			**K**			4	<10	$10^{1.5}$ (1000)	Increased	Comparable
NA-I222R							S			**R**			4	<10	$10^{2.3}$ (150)	Increased	Comparable
NA-R292K			V	M				G			**K**	K/R	8	<10	$10^{4.5}$ (1)	Comparable	Reduced

[a]Mature HA (straight numbering of 2013 H7N9 virus)
[b]Seasonal H3N2 NA numbering (straight 2013 H7N9 NA virus numbering in parenthesis)
[c]MID_{50} and MLD_{50} expressed as $TCID_{50}$
[d]The April 24th specimen was propagated once in chicken eggs (E1) by Taiwan CDC; sequences of this virus isolate were deposited to GISAID (Accession No. EPI445914) and used as reference.
[e]No amino acid differences were detected in the following genes: PB1-F2, NP, M1/M2 and NS2.
WT: wild type; markers of resistance/reduced susceptibility to NA inhibitor(s) are shown in bold.

Table 2. Susceptibility of plaque-purified NA variants of A/Taiwan/1/2013 (H7N9) virus to NA inhibitors

A/Taiwan/1	IC_{50} nM ± SD (fold)[a]				
	Oseltamivir	Zanamivir	Peramivir	Laninamivir	A-315675
NA-WT	0.28 ± 0.02	0.65 ± 0.13	0.08 ± 0.02	0.74 ± 0.16	0.12 ± 0.01
NA-E119V	23.56 ± 4.84 (84)	6.01 ±2.57 (9)	0.10 ± 0.01 (1)	1.31 ± 0.25 (2)	0.14 ± 0.01 (1)
NA-I222K	8.82 ± 0.27 (32)	4.91 ± 0.68 (8)	0.48 ± 0.08 (6)	9.34 ± 1.17 (13)	0.82 ± 0.16 (7)
NA-I222R	10.43 ± 0.62 (37)	7.89 ± 1.82 (12)	0.94 ± 0.13 (12)	10.55 ± 1.06 (14)	0.71 ± 0.08 (6)
NA-R292K	2873 ± 1334 (>10,000)	35.68 ± 4.28 (55)	126.96 ± 22.73 (1587)	16.33 ± 3.19 (22)	1.60 ± 0.11 (13)
Reference viruses A(H3N2)					
A/Washington/01/2007, NA WT	0.07 ± 0.02	0.23 ± 0.03	0.23 ± 0.03	0.29 ± 0.11	0.18 ± 0.07
A/Bethesda/956/2006, NA-R292K	3974 ± 1445 (>10,000)	6.83 ± 1.01 (30)	16.27 ± 0.89 (71)	2.51 ± 0.09 (9)	2.46 ± 0.33 (14)

[a]IC_{50}, a concentration needed to inhibit NA activity by 50%; fold difference compared to IC_{50} of WT virus.
Amino acid substitution in the NA according to N2 amino acid numbering; substitutions correspond to E115V, I219K/R and R289K in the NA of 2013 H7N9 virus. No changes in the NA of WT virus
WHO-AVWG criteria: normal inhibition (<10-fold); reduced inhibition (10−100-fold); highly reduced inhibition (>100-fold)

Figure 1

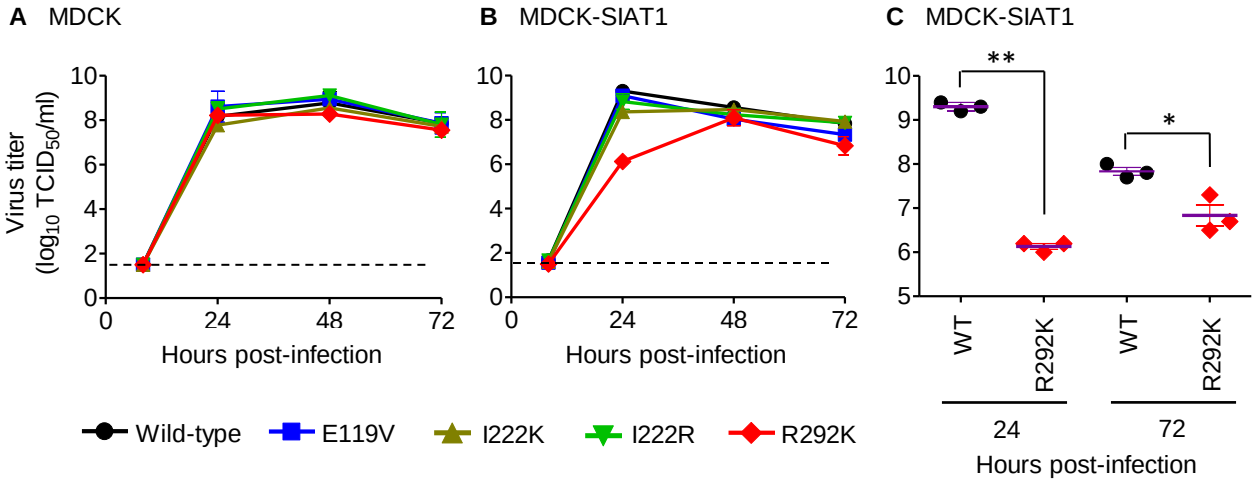

A MDCK
B MDCK-SIAT1
C MDCK-SIAT1
Virus titer
(log10 TCID50/ml)
Hours post-infection
Wild-type
E119V
I222K
I222R
R292K
**
*
WT
R292K
24
72

Figure 2

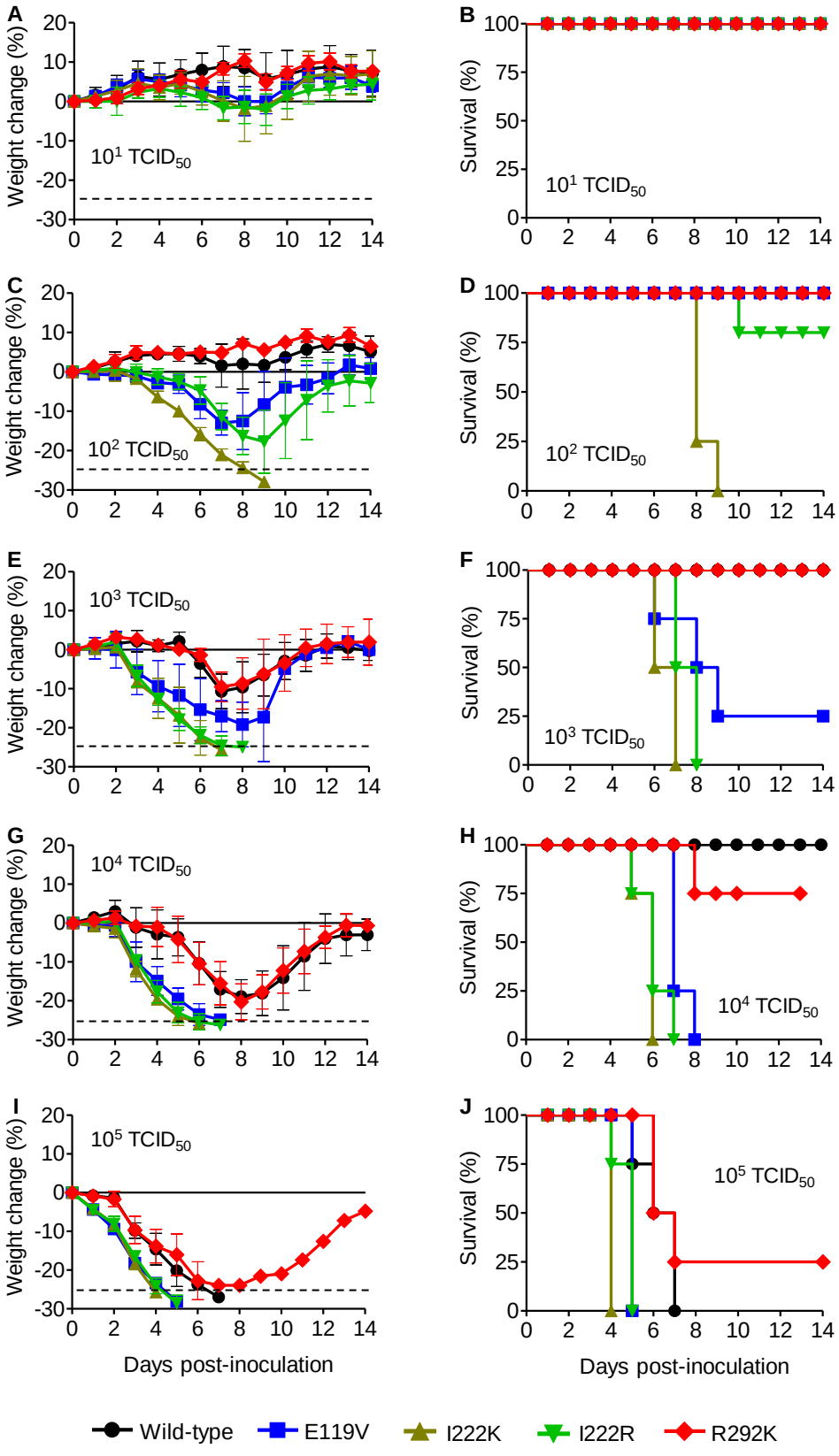
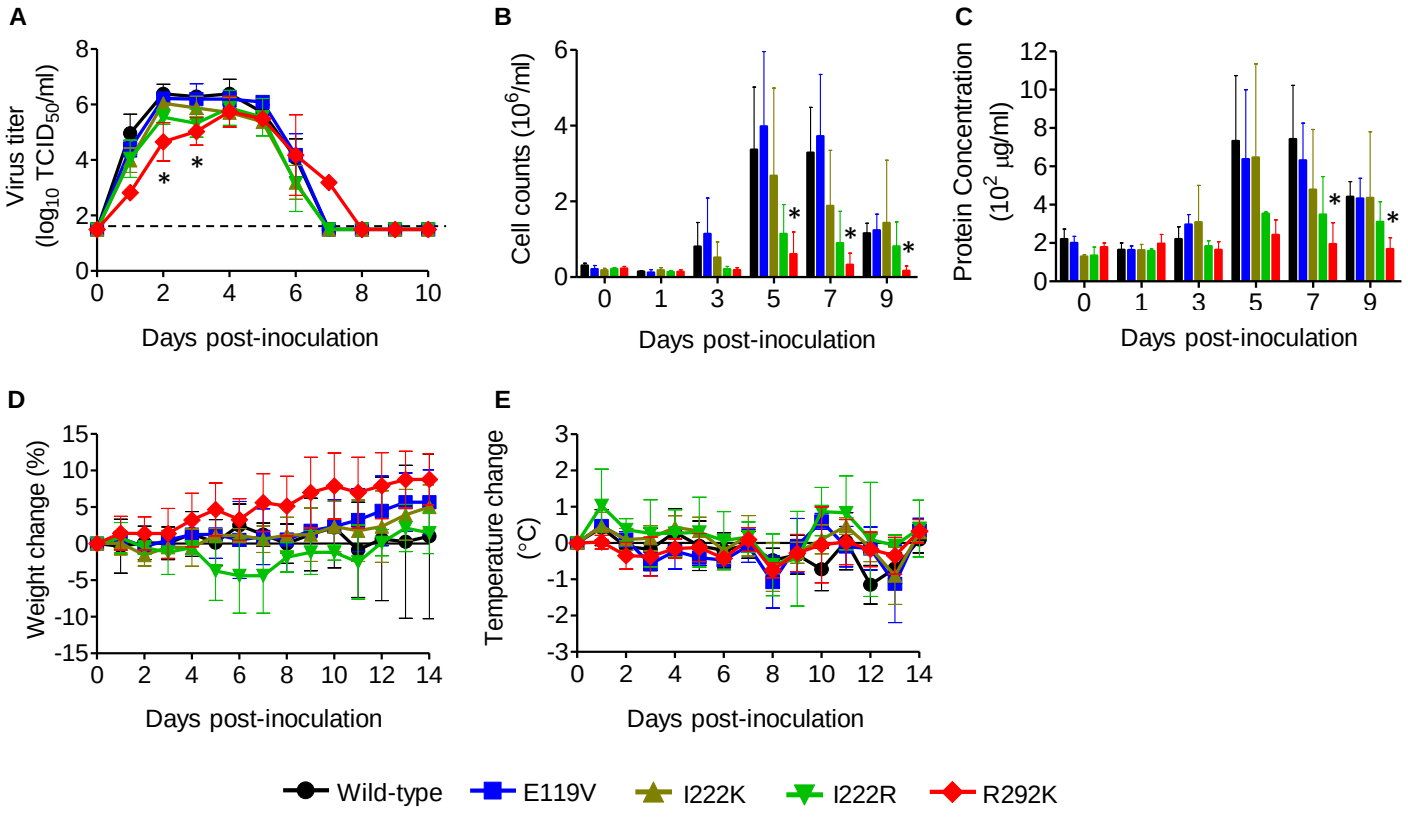

A
Weight change (%)
10¹ TCID50
B
Survival (%)
10¹ TCID50
C
Weight change (%)
10² TCID50
D
Survival (%)
10² TCID50
E
Weight change (%)
10³ TCID50
F
Survival (%)
10³ TCID50
G
Weight change (%)
10⁴ TCID50
H
Survival (%)
10⁴ TCID50
I
Weight change (%)
10⁵ TCID50
J
Survival (%)
10⁵ TCID50
Days post-inoculation
Days post-inoculation
Wild-type
E119V
I222K
I222R
R292K

Figure 3


A
Virus titer
(log_{10} $TCID_{50}$/ml)
Days post-inoculation
B
Cell counts (10^6/ml)
Days post-inoculation
C
Protein Concentration
(10^2 µg/ml)
Days post-inoculation
D
Weight change (%)
Days post-inoculation
E
Temperature change
(°C)
Days post-inoculation
Wild-type
E119V
I222K
I222R
R292K