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Low replicative fitness of neuraminidase inhibitor-resistant H7N9 avian influenza A virus with R292K substitution in neuraminidase in cynomolgus macaques compared with I222T substitution

Running title: Low replication of drug-resistant H7N9 influenza virus

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Abstract

Human cases of H7N9 influenza A virus infection have been increasing since 2013. The first choice of treatment for influenza is neuraminidase (NA) inhibitors (NAIs), but there is a concern that NAI-resistant viruses are selected in the presence of NAIs. In our previous study, an H7N9 virus carrying AA substitution of threonine (T) for isoleucine (I) at residue 222 in NA (NA222T, N2 numbering) and an H7N9 virus carrying AA substitution of lysine (K) for arginine (R) at residue 292 in NA (NA292K, N2 numbering) were found in different macaques that had been infected with A/Anhui/1/2013 (H7N9) and treated with NAIs. In the present study, the variant with NA292K showed not only resistance to NAIs but also lower replication activity in MDCK cells than did the virus with wild-type NA, whereas the variant with NA222T, which was less resistant to NAIs, showed replication activity similar to that of the wild-type virus. Next, we examined the pathogenicity of these H7N9 NAI-resistant viruses in macaques. The variants caused clinical signs similar to those caused by the wild-type virus with similar replication potency. However, the virus with NA292K was replaced within 7 days by that with NA292R (same as the wild-type) in nasal samples from macaques infected with the virus with NA292K, i.e. the so-called revertant (wild-type virus) became dominant in the population in the absence of an NAI. These

results suggest that the clinical signs observed in macaques infected with the NA292K virus are caused by the NA292K virus and the NA292R virus and that the virus with NA292K may not replicate continuously in the upper respiratory tract of patients without treatment as effectively as the wild-type virus.

Keywords: H7N9 avian influenza virus; nonhuman primates; neuraminidase inhibitor; resistance; reverse genetics; revertant

Abbreviations

AA: amino acid

Anhui/1: A/Anhui/1/2013 (H7N9)

BSA: bovine serum albumin

CPE: cytopathic effect

DMEM: Dulbecco's modified Eagle's medium

EC₅₀: half maximal effective concentration

EMEM: Eagle's minimal essential medium

IC₅₀: half maximal inhibitory concentration

HA: hemagglutinin

HBSS: Hanks buffered saline solution

H&E: hematoxylin and eosin

MDCK: Madin-Darby canine kidney

m.o.i.: multiplicity of infection

NA: neuraminidase

NAIs: neuraminidase inhibitors

PFU: plaque-forming unit

rg: reverse genetics

TCID₅₀: 50% tissue culture infective dose

1. Introduction

H7N9 avian influenza A virus infection in humans has been reported since 2013 in China. After the occurrence of the greatest epidemic wave in the season of 2016-2017, 1568 cases including 615 deaths were reported up to November 2019 (WHO, 2019). Some patients infected with the H7N9 virus showed severe clinical symptoms with pneumonia and acute respiratory distress syndrome (Gao et al., 2013; Hu et al., 2013). In our previous study, the efficacy of neuraminidase inhibitors (NAIs) against the H7N9 influenza virus A/Anhui/1/2013 (H7N9) (Anhui/1) was examined in cynomolgus macaques (Itoh et al., 2015). Although oseltamivir and peramivir reduced the virus titers in macaques, variants with I222T and R292K in neuraminidase (NA) [N2 numbering, amino acid (AA) positions 219 and 289 in the N9 numbering of A/Anhui/1/2013 (H7N9)] were detected in different macaques treated with NAIs. R292K in NA has been identified in human virus samples (Hu et al., 2013; Lin et al., 2014; Sleeman et al., 2013; Marjuki et al., 2015), whereas I222T among the N9 subtype was identified for the first time in our previous study (Itoh et al., 2015). Other non-I222T substitutions at NA222 in the N9 subtype were found in human samples (Marjuki et al., 2015), and I222T was found in H5N1 and influenza B viruses (McKimm-Breschkin et al., 2013; Monto et al., 2006). According to the results of molecular

dynamics simulations, the substitution of R292K directly reduced NAI binding, whereas the substitution of I222T indirectly changed the conformation of the catalytic site of NA (Itoh et al., 2015).

NAIs are used for treatment of patients infected with influenza A and B viruses. In H1N1 and H3N2 seasonal influenza viruses, NA variants resistant to NAIs (H274Y, R292K) showed lower replication potency (replicative fitness) in ferret models and *in vitro* (Herlocher et al., 2004; Ives et al., 2002) than did NAI-sensitive viruses, whereas an H1N1 resistant virus (H275Y) dominated the viruses sensitive to NAIs a few seasons later in Japan (Ujike et al., 2010). The H3N2 virus with reduced-susceptibility (I222T/S331R or R292K), which is sporadically detected, has been a minor population in circulating viruses in humans (Hurt et al., 2016). In addition, it has been speculated that mutations in not only NA genes but also HA genes are responsible for fitness for replication of viruses with NAI resistance (Govorkova, 2013). Although the characteristics of H7N9 variants have been evaluated in mice, guinea pigs, and ferrets (Zhang et al., 2014; Hai et al., 2013; Baranovich et al., 2014; Yen et al., 2014), the pathogenicity and replication efficiency of NAI-resistant variants in non-human primates, which are physiologically and genetically similar to humans, have not been revealed.

In the present study, we examined the replicative fitness and pathogenicity of variant H7N9 viruses with NA222T or NA292K in cell culture and in macaques without NAI treatment. Low replication ability of the virus carrying NA292K was confirmed *in vitro*. The macaques infected with the H7N9 viruses with NA222T or NA292K showed significant symptoms as did macaques infected with the wild-type virus Anhui/1 (Itoh et al., 2015). After infection, the percentage of the variant virus carrying NA222T did not change in the virus population, whereas the variant carrying NA292K decreased in the population and the virus with NA292K was replaced by the virus with NA292R (wild-type). Therefore, the virus carrying NA292K, which showed high resistance to NAIs, may not replicate efficiently in the upper respiratory tract of untreated patients in the absence of selective pressure of NAIs, but the virus carrying NA292K may replicate in the lower respiratory tract.

2. Materials and Methods

2.1. Ethics statement

This study was carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga

University of Medical Science and Standards Relating to the Care and Management, etc. of Experimental Animals (Notification No. 6, March 27, 1980 of the Prime Minister's Office, Japan). The protocol was approved by the Shiga University of Medical Science Animal Experiment Committee (permission 2016-6-2). Regular veterinary care and monitoring, balanced nutrition and environmental enrichment were provided by the Research Center for Animal Life Science at Shiga University of Medical Science. The macaques were euthanized at the endpoint 7 days after virus inoculation using ketamine/xylazine followed by intravenous injection of pentobarbital (200 mg/kg). The animals were monitored every day during the study to be clinically scored as shown in Table S1 and to undergo veterinary examinations to help alleviate suffering. Animals would be euthanized if their clinical scores reached 15 (a humane endpoint), although no animals showed symptoms scored as 15 in the present study (Pham et al., 2013; Itoh et al., 2015).

2.2. Animals

The macaques used in this study were free from herpes B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella spp.*, *Salmonella spp.*, and *Entamoeba histolytica*. Female cynomolgus macaques (5 – 9 years of age) in the Research Center for Animal Life Science, Shiga

University of Medical Science that had been bred from macaques originating in Indonesia were healthy adults. Sample collection and virus inoculation were performed under conditions of ketamine (5 mg/kg) and xylazine (1 mg/kg) anesthesia, and all efforts were made to minimize suffering. Food pellets of CMK-2 (CLEA Japan, Inc., Tokyo, Japan) were given once a day after recovery from anesthesia, and drinking water was available *ad libitum*. The animals were singly housed under conditions of controlled humidity (47% to 54%), temperature (23 to 24 °C), and light (12-h light/12-h dark cycle; lights on at 8:00 a.m.). In the text and figures, individual macaques are distinguished by the following identification numbers: T1, T2, and T3 as macaques infected with A/Anhui/1/2013 with NA variants I222T (NA222T) and K1, K2, and K3 as macaques infected with A/Anhui/1/2013 with NA variants R292K (NA292K). Two weeks before virus inoculation, a telemetry probe (TA10CTA-D70; Data Sciences International, St. Paul, MN) was implanted in the peritoneal cavity of each macaque under the condition of ketamine/xylazine anesthesia followed by isoflurane inhalation to monitor body temperature. Under the condition of ketamine/xylazine anesthesia, two cotton sticks (TE8201, Eiken Chemical, Ltd., Tokyo, Japan) were used to collect fluid samples in nasal cavities, oral cavities, and tracheas every day from day 0 to day 7, and the sticks were subsequently immersed in 1 mL of Eagle's minimal essential medium (EMEM)

containing 0.1% bovine serum albumin (BSA) and antibiotics. A bronchoscope (MEV-2560; Machida Endoscope Co. Ltd., Tokyo, Japan) and cytology brushes (BC-203D-2006; Olympus Co., Tokyo, Japan) were used to obtain bronchial samples.

2.3. Viruses and cells

Influenza virus A/Anhui/1/2013 (H7N9) (Anhui/1, kindly provided by Eri Nobusawa, Kazuya Nakamura and Masato Tashiro, National Institute of Infectious Disease, Japan) was isolated from a human patient (Gao et al., 2013; Kageyama et al., 2013) and propagated in chicken embryonated eggs once at 35°C for 48 h at the Shiga University of Medical Science (Itoh et al., 2015; Shichinohe et al., 2016). Virus isolates with NA222T or NA292K were detected in nasal samples from separate Anhui/1-inoculated and NAI-treated macaques in the previous study (Itoh et al., 2015). Since the viruses were a mixture of wild-type and NA variants, we isolated these viruses in plaque purification using Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection, Manassas, VA) after staining the plaques with neutral red. After collecting the plaques, we propagated the viruses by using MDCK cells and stored them at -80 °C until use. The changes at NA222T and NA292K were detected in genes from the nasal swab samples before

culture of MDCK cells as described in the previous study (Itoh et al., 2015). Three macaques were challenged with NA222T virus and three other macaques were challenged with NA292K virus (3×10^6 plaque-forming units [PFU] in 7 mL Hanks buffered saline solution [HBSS]). The virus was inoculated into the conjunctivas (0.05 mL for each conjunctiva), nostrils (0.5 mL for each nostril), oral cavity (0.9 mL), and trachea (5 mL) using pipettes and catheters. For virus titration, serial dilutions of nasal swabs, oral swabs, tracheal swabs, and bronchial swabs were inoculated onto confluent MDCK cells. The MDCK cells were then cultured in MEM including 0.1% BSA and trypsin (5 μ g/mL). The presence of cytopathic effects (CPE) was determined under a microscope 72 h later, and the virus concentration (TCID₅₀/mL) was calculated (Itoh et al., 2008).

To compare the replicative fitness between *in vivo* and *in vitro*, we passaged viral stock (NA222T and NA292K) in MDCK twice (Table S5). The virus with NA222T and that with NA292K were cultured in MDCK cells at a multiplicity of infection (m.o.i.) of 0.01 for 48 h until showing CPE, and supernatants were collected and stored at -80 °C. Then virus titers in the collected supernatants were sequenced and the viruses were passaged twice in the same condition.

2.4. Plasmids and reverse genetics

Plasmid-based reverse genetics for influenza virus generation was performed (Hoffmann et al., 2000; Soda et al., 2011). pHW2000 plasmids encoding the complementary DNAs for eight segments of Anhui/1 RNA were constructed. Plasmids encoding Anhui/1, HA with substitution of leucine to glutamine at 226 (HA226Q, H3 numbering), NA with substitution of isoleucine to threonine at 222 (NA222T) and NA with substitution of arginine to lysine at 292 (NA292K) under control of the CMV promoter were transfected. For transfection, 293T cells and MDCK cells were co-cultured in OPTI-MEM (Thermo Fisher SCIENTIFIC, MA, USA) in a six-well plate for one day at 37 °C. Six combinations of plasmids coding NA and HA (wild-type NA + wild-type HA226L, NA222T + HA226L, NA292K + HA226L, wild-type NA + HA226Q, NA222T + HA226Q, NA292K + HA226Q) together with other segments (PB2, PB1, PA, NP, M, and NS) were mixed with OPTI-MEM and TransIT-293 (Mirus Bio LCC., WI, USA) for 30 min at room temperature and then added into the plates. After incubating for 6 h at 37 °C, the medium was changed to new OPTI-MEM and incubated at 37 °C again. After incubation for 30 h, new OPTI-MEM containing trypsin (5 µg/mL) was added and cultured at 35 °C for four to 6 days, and then culture supernatants were collected and inoculated into MDCK cells for virus propagation. All six viruses made by reverse genetics including rgAnhui/1 (virus with a gene constellation of the wild-

type Anhui/1) had identical nucleotide changes in the HA gene, which coded the AA sequence FVSGSK at residues 309 to 314 instead of PFQNID as detected in wild-type Anhui/1.

2.5. Growth kinetics of virus in cell culture and drug resistance assay

To examine the propagation of mutant viruses generated by reverse genetics, MDCK cells and A549 cells (human lung carcinoma cells obtained from RIKEN RBC through the National Bio-Resource Project of MEXT, Japan) were cultured in 12-well plates for two days until becoming confluent. The six variant viruses were inoculated into the cells at an m.o.i. of 0.01. After incubation at 35 °C for 1 h, the cells were washed with HBSS once, and then EMEM containing 0.1% BSA with trypsin (5 µg/mL) and Dulbecco's modified Eagle's medium (DMEM) containing 0.3% BSA and trypsin (5 µg/mL) for MDCK cells and A549 cells, respectively, were added and the cells were cultured at 35 °C. Culture supernatants were collected at 6, 12, 24, 48, and 72 h after infection to measure virus titers.

To examine antiviral drug susceptibility of NAI-resistant variants, MDCK cells and A549 cells were cultured with viruses for 1 h at an m.o.i. of 0.01. MDCK cells and A549 cells were washed with HBSS and cultured in EMEM containing 0.1% BSA and trypsin (5 µg/mL) and

DMEM containing 0.3% BSA and trypsin (5 µg/mL), respectively, with or without oseltamivir acid (Chemscene Ltd., NJ) or peramivir hydrate (Shionogi & Co., Ltd., Osaka, Japan) (0 – 10 µg/mL) for 24 or 48 h at 35 °C. Culture supernatants were collected to measure virus titers.

2.6. NA inhibition assay

Half maximal inhibitory concentrations (IC_{50}) of oseltamivir and peramivir against enzymatic activity of NA of the viruses generated by reverse genetics were determined as previously described (Leang et al., 2017). Briefly, diluted viruses were mixed with the indicated concentrations of oseltamivir or peramivir and incubated at 37 °C for 30 min. 2'-(4-Methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUNANA) substrate (Nacalai Tesque, Kyoto, Japan) was then added as a fluorescent substrate, and the mixture was incubated at 37 °C for 1 h. The reaction was stopped by adding 0.14 M NaOH in ethanol. The fluorescence of the solution was measured at an excitation wavelength of 365 nm and an emission wavelength of 415 – 445 nm, and the IC_{50} values were calculated.

2.7. Sequence analysis of NA and HA genes and allele frequency analysis by deep sequencing

Viral RNA was extracted from supernatants or suspensions of swab samples using a Qiagen viral RNA minikit (Qiagen, Hilden, Germany) and reverse-transcribed with Uni12 primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen Corporation, Carlsbad, CA) (Desselberger et al., 1980; Itoh, et al. 2015). The partial NA and HA regions of the influenza virus were amplified using the primer sets of forward primer 5'-TGCACTTCAGCCACTGCTAT-3' and reverse primer 5'-ATATCGTCTCGTATTAGTAGAAACAAGGGTCTT-3' and forward primer 5'-CCTGGTATTCGCTCTGATTGC-3' and reverse primer 5'-TGCTACCAAGAGTTCAGCATTG-3', respectively. For amplification by KOD plus-neo DNA polymerase (Toyobo Co. Ltd., Osaka, Japan), after denaturation at 94 °C for 2 min, the reaction was performed with 30 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 68 °C for 45 s. For amplification by PrimeSTAR Max DNA polymerase (Takara Bio Inc.), after denaturation at 98 °C for 2 min, the reaction was performed with 35 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 1 min.

PCR amplicons were sheared to make the length about 300 bp using Covaris S220 (Covaris Inc., MA) and libraries were prepared by using a KAPA Hyper Prep Kit (KAPA Biosystems) according to the manufacturer's protocol. The 251 bp paired-end sequencing was

performed using a MiSeq v2 500 cycle kit (Illumina Inc., CA), and about 90,000 - 130,000 reads were obtained for each sample. Raw reads for each sample were mapped onto the reference sequence using bowtie2, and count files were generated using igvtools.

3. Results

3.1. Low replication rate of the H7N9 virus with NA292K/HA226Q in cells

In our previous study (Itoh et al., 2015), two variant viruses were isolated from nasal swab samples of different cynomolgus macaques that had been infected with wild-type virus Anhui/1 and treated with NAIs: one was a virus with a substitution at AA position 222 in NA (NA222T), and the other was a virus with a substitution at AA position 292 in NA (NA292K). In our previous study, the both viruses showed higher IC_{50} values against NAIs than did Anhui/1. To examine the effect of each substitution in the NA on virus replication, we generated viruses carrying substitutions in NA using reverse genetics (rg). At cloning and confirming of viral genes, we detected several substitutions in HA (Table S2). Among them, the virus with NA222T and the virus with NA292K had a substitution of glutamine at 226 in HA (L226Q) (residue 226 in H3 numbering corresponding to 235 in H7 numbering), which is located in the sialic acid-binding

pocket (Rogers et al., 1983), although the wild-type Anhui/1 possessed leucine at residue 226 in HA (HA226L). Finally, six variants were constructed by combinations of three mutations coding NA222T, NA292K, and HA226Q. Virus titers of all variants reached plateau levels after 24 h in culture of MDCK cells (Fig. 1A) and after 48 h in culture of A549 cells (Fig. S1A). The virus carrying NA292K together with HA226Q (rgNA292K/HA226Q) showed lower virus titers than did the other variants in MDCK cells (Fig. 1A). On the other hand, in human lung carcinoma A549 cells, NA292K/HA226Q showed a slightly lower growth rate than did rgAnhui/1 with HA226L (wild-type) (Fig. S1A). Three AAs at NA222, NA292, and HA226 did not change after 72 h in culture, as confirmed by nucleotide sequence analysis (data not shown). The results suggest that rgNA292K/HA226Q has lower replication potency than do other viruses *in vitro* although they have a similar replication potency in human cells.

3.2. High resistance of H7N9 viruses with NA292K to oseltamivir and peramivir

We examined the efficacy of anti-influenza drugs against the variant viruses. MDCK and A549 cells were infected and treated with NAIs (oseltamivir and peramivir, 0.001 – 10 µg/mL), and then viral titers were measured at 24 h and 48 h (Fig. 1B, C and Fig. S1B, C). The virus titers

of rgAnhui/1 (wild-type NA/HA226L) and rgNA222T/HA226L were decreased in the presence of oseltamivir and peramivir, and rgHA226Q and rgNA222T/HA226Q were more sensitive to the NAIs than rgAnhui/1 and rgNA222T/HA226L (Fig. 1B, C). The half maximal effective concentrations (EC_{50} values) for oseltamivir and peramivir against all strains were calculated in MDCK and A549 cells. The rgHA226Q and rgNA222T/HA226Q strains had lower EC_{50} values for oseltamivir and peramivir than did rgAnhui/1 and rgNA222T/HA226L strains (Table S3). We also examined the half maximal inhibitory concentrations (IC_{50} values) against NA enzymatic activity for all the viral strains (Table 1). rgAnhui/1 and rgHA226Q exhibited similar IC_{50} values to each other, as did rgNA222T/HA226L and rgNA222T/HA226Q (Table 1). There was virtually no decrease in viral titers for rgNA292K/HA226L and rgNA292K/HA226Q in the presence of the NAIs (Fig. 1B, C, Fig. S1B, C). However, rgNA292K/HA226Q had slightly higher EC_{50} and IC_{50} values relative to rgNA292K/HA226L. These results suggest that the NA292K substitution is responsible for resistance to NAIs. It is also suggested that the HA226Q substitution has a positive effect on the replicative fitness of viruses with wild type NA and NA222T and a negative effect on that of the virus with NA292K. Collectively, these results suggest that the substitution of NA292K is critical for escape from NAIs in macaques.

3.3. Disease signs and viral pneumonia in macaques infected with two NAI-resistant H7N9 variants

To compare the pathogenicity of NA222T/HA226Q and NA292K/HA226Q viruses to that of Anhui/1, macaques were infected with plaque-purified NA222T/HA226Q or NA292K/HA226Q viruses. The body temperatures of the macaques rose within 24 h after virus inoculation and did not return to the basal level during the study period as was previously observed in macaques inoculated with Anhui/1 (Fig. 2A, Fig. S2) (Itoh et al., 2015). Body weights decreased, but the changes were not statistically significant (Fig. 2B). Clinical scores in macaques infected with NA222T/HA226Q or NA292K/HA226Q viruses increased after virus inoculation due to the increase in body temperature and loss of appetite (Fig. 2C, Table S1). No other signs of disease were observed.

We examined inflammation caused by infection and viral cell tropism histologically in lung tissues of the macaques that had been infected with each of the two variants. Alveolar spaces were filled with exudate, neutrophils, and lymphocytes in the macaques infected with either variant viruses (Fig. 3A, B) as well as in a macaque infected with Anhui/1 (Fig. 3C) that we reported

previously (Itoh et al., 2015) but not in an uninfected control macaque (Fig. 3D). The influenza A virus nucleoprotein was detected in type-I alveolar epithelial cells and type-II alveolar epithelial cells (Fig. 3E-G). Thus, the two variants caused viral pneumonia as did the wild-type virus.

3.4. Virus propagation in macaques infected with the two NAI-resistant H7N9 variants

We examined virus titers in swab and tissue samples to verify virus propagation. The virus was detected in nasal, oral, tracheal, and bronchial samples of the macaques in both groups from day 1 to day 6 or 7 after virus infection (Fig. 4A-D, Table S4). Virus titers in the samples from macaques in the NA222T/HA226Q and NA292K/HA226Q groups were comparable to those in the samples from macaques in the Anhui/1 group except for in bronchial samples (Table S4, Itoh et al., 2015). In bronchial samples, the virus titers in macaques infected with NA222T/HA226Q on day 4 and day 7 and that in infected with NA292K/HA226Q on day 7 were significantly higher than those in macaques infected with the wild-type virus. The virus titers in the samples from macaques in the NA222T/HA226Q group were higher than those in the samples from macaques in the NA292K/HA226Q group, especially oral samples at day 1 and day 7 of NA222T/HA226Q group were significantly higher than that of NA292K/HA226Q group (Fig. 4B). The virus was

detected in respiratory tissues 7 days after infection (Fig. 4E). The virus titers of NA222T/HA226Q in tissue samples were higher than those of NA292K/HA226Q especially in tracheas, conjunctivas, and a part of the lung.

3.5. Increases of the virus with wild-type NA292R in nasal samples from macaques infected with H7N9 virus with NA292K/HA226Q

The NA genes of viruses in nasal swab samples from the macaques infected with the two variant viruses were examined by deep sequencing. After infection with NA222T/HA226Q, threonine at AA position 222 was maintained predominantly on days 5 and 7 in the nasal samples from macaques infected with NA222T/HA226Q (Table 2). In contrast, the virus carrying arginine (R) at position 292 increased in nasal swab samples instead of the virus carrying lysine (K), so revertant (wild-type) became dominant (Table 3). The percentages of other mutations in the NA gene were up to 12% in one macaque (T2) (data not shown). On the other hand, in lung samples collected on day 7, NA222T and NA292K were major populations in macaques inoculated with NA222T/HA226Q and NA292K/HA226Q, respectively (Fig. S3). Therefore, population change was dependent on the organ or tissue. After three *in vitro* passages in MDCK cells, no increase in

the percentage of wild-type sequences in NA genes was detected in culture with NA222T/HA226Q or NA292K/HA226Q (Table S5). The results suggest that NA222T substitution does not affect the replicative fitness of the virus since the percentage of the virus with NA222I (wild-type) was less than 1% after one passage in macaques, whereas the virus carrying NA292K substitution has a disadvantage in replication in macaques without NAI treatment compared with that carrying NA292R (wild-type). This result indicates that the clinical signs seen in the macaques infected with NA292K/HA226Q were caused by NA292K/HA226Q and NA292R/HA226Q viruses in the later time point of infection.

3.6. Sensitivity of the mixed population of NA292K and NA292R viruses to oseltamivir

To confirm the susceptibility of the virus population propagated in the macaques to NAIs, we examined the replication potency of viruses that were recovered from macaques K1 and K3 as representative viruses (The remaining samples of K2 were insufficient for analysis.) after one passage in MDCK cell culture. After the passage, the percentages of NA292K in the passaged samples from K1 on day 1 and day 7 were 99.68% and 0.11%, respectively, and those of NA292K in the passaged samples from K3 on day 1 and day 7 were 99.47% and 13.58%, respectively (Table

S6). Replication of the virus recovered from K1 on day 1 was not inhibited by oseltamivir (0.001 – 10 µg/mL) in the culture fluid of MDCK cells for 24 h, whereas replication of the virus recovered on day 7 was inhibited by oseltamivir as was seen for Anhui/1 (wild-type) (Fig. 5). Although the percentage of NA292K in virus samples from K3 on day 7 after a passage *in vitro* was lower than that of the original viral population (26.56% as shown in Table 1), the replication of viruses recovered from K3 on days 1 and 7 was not inhibited by oseltamivir as was the case for NA292K virus (inoculum). Since the difference in viruses recovered on day 7 from K1 and K3 was the percentages of virus with NA292K in the population (0.11% in K1 and 13.58% in K3), it was thought that an increase of the virus with NAI-sensitive NA292R directly restored susceptibility of the virus population to NAIs.

4. Discussion

Using cell culture and cynomolgus macaques, we revealed the replication and pathogenicity of two H7N9 avian influenza viruses with AA substitutions in the NA protein were less sensitive to NAIs than was the wild-type strain Anhui/1 (Itoh et al., 2015). To examine the effects of AA substitutions of NA222T, NA292K and HA226Q on viral replication, we constructed

viruses with combinations of mutations in the NA and HA genes using reverse genetics. rgNA292K/HA226Q showed lower virus titers than those of the other variants in culture of MDCK cells, whereas rg viruses with NA222T showed virus titers similar to those of rg viruses with wild-type NA. In the macaque model, NA222T/HA226Q and NA292K/HA226Q viruses caused raised body temperature and loss of appetite, and virus propagation was observed in swab samples and respiratory tissue samples from macaques infected with either virus. Unlike the results obtained in an *in vitro* study, deep sequencing revealed that the virus carrying NA292K was replaced by that carrying NA292R (wild-type) during infection in nasal samples from macaques without NAI treatment, whereas the virus carrying NA222T was detected predominantly throughout the infection. These results showed that the two NAI-resistant H7N9 avian influenza viruses with NA222T and NA292K substitutions have different replication rates that result in population stability and population change, respectively.

The clinical signs seen in the macaques infected with NA292K/HA226Q virus were similar to those seen in the macaques infected with wild-type Anhui/1. However, deep sequence analysis revealed that the percentage of NA292R/HA226Q virus increased in nasal samples during the infection, although NA292R/HA226Q virus did not increase in *in vitro* passages and in the lung

tissues. The reasons for population stability are thought to be a death of MDCK cells in culture before a change of virus population and an environment of the lung tissue, i.e. lack of mucus produced by submucosal glands, which are located from the nasal mucosa to the bronchus (Yen et al., 2014), and higher temperature in the lung than the nasal cavity, in which the NA folding efficacy and viral replication decreased (da Silva et al., 2015). Therefore, the replication rate of the virus with NA292K in the nasal cavity was lower than that of the virus with NA292R, and the symptoms seen in macaques infected with NA292K/HA226Q virus might be caused by both NA292K/HA226Q virus and NA292R/HA226Q virus. On the other hand, low virus titers of rgNA292K/HA226Q in the culture of MDCK cells are consistent with the population change with increase in the percentage of NA292R/HA226Q virus in swab samples in the absence of NAIs (Yen et al., 2014). Furthermore, the results suggest expansion of NA292R/HA226Q virus in the inoculum (0.11%) during replication and/or shedding in nasal cavity of macaques without treatment, although no reversion was found in the lung tissue and a ferret model (Marjuki et al., 2015). Therefore, a virus that is highly resistant to NAIs such as NA292K/HA226Q virus may not be a major virus population in an upper respiratory tract of patients without NAI treatment (Treurnicht et al., 2019).

The pathogenicity of NA222T/HA226Q virus in macaques was similar to that of wild-type Anhui/1 (Itoh et al., 2015). In addition, substitution of the AA at position 222 of NA did not change the viral replication, as was confirmed in the *in vitro* study. Since the percentage of virus carrying NA222I (wild-type) slightly increased in macaques for 7 days, the results indicate the possibility that the virus with NA222I (wild-type) replaces that with NA222T after further passages. However, since rgNA222T/HA226Q and rgHA226Q viruses were more sensitive to NAIs than was the virus with HA226L, there might not be a threat of expansion of NA222T/HA226Q virus during treatment with NAIs. In patients without NAI treatment, NA222T/HA226Q virus might propagate as much as the wild-type virus, suggesting a possibility that NA222T/HA226Q virus coexists in patients.

The substitution HA226Q was shown to affect virus propagation and NAI sensitivity in the cell culture study. In the absence of NAIs, rgNA292K/HA226Q virus showed lower replication potency than did rgNA292K/HA226L, whereas rgNA292K/HA226Q virus showed the greatest replication rate in the presence of NAIs among the rg viruses examined. In addition, rgNA292K/HA226Q virus showed a tendency to be more resistant than rgNA292K/HA226L virus to NAIs at a high concentration. Thus, HA226Q reduced the replication rate of the virus with

NA292K. On the other hand, in rgAnhui/1 and rgNA222T viruses, the virus with HA226Q substitution was more sensitive than the virus with HA226L substitution to NAIs, although propagation of the viruses was not affected in the absence of NAIs. Therefore, AA substitution of HA226Q works as an NAI-resistant factor when a virus has an NA that is highly resistant to NAIs, whereas HA226Q works as an NAI-sensitive factor when a virus has an NA that is sensitive or less resistant to NAIs. Therefore, we consider that the replicative fitness and drug resistance are affected by the balance of function between HA and NA proteins (Gaymard et al., 2016; Lai et al., 2019).

In summary, we examined the pathogenicity of NA222T/HA226Q and NA292K/HA226Q viruses in untreated macaques. We also examined replication kinetics of viruses with various combinations of AA substitutions and sensitivities of the viruses to antiviral drugs *in vitro*. The virus carrying NA292K was gradually replaced by the virus carrying NA292R (same as the wild-type) in nasal samples from the macaques without treatment. Therefore, the virus carrying NA292K might not become a major population in the upper respiratory tract of patients without treatment, although the virus carrying NA292K might propagate in the lower respiratory tract. In addition, the virus carrying NA222T might show potential to replicate at the same rate as that of the wild-type virus.

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Figure legends

Fig 1. Replication and sensitivity to antiviral drugs of variant viruses carrying NA222T, NA292K, and HA226Q in MDCK cells.

(A) Variant viruses carrying HA226L, HA226Q, wild-type NA, NA222T, or NA292K were inoculated into culture of MDCK cells at an m.o.i. of 0.01 to determine propagation rates. (B, C) MDCK cells were cultured with viruses for 1 h at an m.o.i. of 0.01, and then the viral inoculum was replaced by a medium containing oseltamivir acid (B) or peramivir hydrate (C), at a concentration between 0 – 10 µg/mL. Culture supernatants were collected after 24 h for virus titration. Averages and standard deviations of hexaplicates are shown. Asterisks indicate significant differences compared with rgAnhui/1 (HA226L, wild-type) (* $P < 0.05$, ** $P < 0.01$, Mann-Whitney U test).

Fig 2. Clinical symptoms of macaques infected with NA222T/ HA226Q and NA292K/ HA226Q viruses.

Cynomolgus macaques (n = 3 in each group) were inoculated with the influenza viruses NA222T/HA226Q and NA292K/HA226Q viruses, which are NAI-resistant variants originating

from A/Anhui/1/2013 (H7N9). (A) Average body temperatures from 8 p.m. to 8 a.m. on the next day were calculated for individual macaques (Fig. S2). Average body temperatures on each day were compared with those from 8 p.m. on the day before virus inoculation (day -1) to 8 a.m. on the day of virus inoculation (day 0). (B) Body weights on each day were compared with that on day 0 before virus inoculation. (C) Clinical scores were determined by daily observation and body temperature according to Table S1. Average and standard deviations of the results of three monkeys are shown. Red: macaques inoculated with NA222T/HA226Q, blue: macaques inoculated with NA292K/HA226Q. An asterisk indicates a significant difference between the two groups on day 5 ($P < 0.05$, Mann-Whitney U test).

Fig 3. Viral pneumonia in macaques infected with NA222T/HA226Q and NA292K/HA226Q viruses.

All macaques infected with virus were autopsied 7 days after virus inoculation. Representative photos for each group are shown. (A, E) Lung tissues of macaques infected with NA222T/HA226Q (T1). (B, F) Lung tissues of macaques infected with NA292K/HA226Q (K2). (C, G) Lung tissues of macaques infected with Anhui/1 in our previous study (Itoh et al., 2015). (D, H) Lung tissues of

a normal uninfected macaque. New sections were prepared from the paraffin blocks made in the previous study (Itoh et al., 2015) and stained (C, G, D, H). (A-D) H & E staining. (E-H) and immunohistochemical staining for influenza virus NP (brown). White arrowheads: type-I alveolar epithelial cells, black arrowheads: type-II alveolar epithelial cells, bars: 50 μ m.

Fig 4. Virus titers in swab and tissue samples from macaques infected with NA222T/HA226Q and NA292K/HA226Q.

Cynomolgus macaques were inoculated with NA222T/HA226Q and NA292K/HA226Q viruses on day 0. (A-D) Nasal, oral, tracheal, and bronchial samples were collected on the indicated days.

Averages and standard deviations of virus titers in the nasal (A), oral (B), tracheal (C), and bronchial (D) samples were calculated on the basis of individual titers listed in Table S4. (E)

Averages of virus titers in tissue samples. The samples were collected at autopsy 7 days after virus inoculation. R: right, L: left, RU: right upper lobe, RM: right middle lobe, RL: right lower lobe,

LU: left upper lobe, LM: left middle lobe, LL: left lower lobe. The detection limit of virus titer is 0.67 \log_{10} TCID₅₀/mL in swab samples and 1.67 \log_{10} TCID₅₀/g in tissue samples. Virus titers under the detection limit were calculated as 0. Averages and standard deviations of the results for three

macaques are shown. Red: macaques inoculated with NA222T/HA226Q, blue: macaques inoculated with NA292K/HA226Q. Asterisks indicate significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

Fig 5. Viral sensitivity to oseltamivir of viruses isolated from nasal samples in MDCK cells.

Anhui/1 (wild-type), NA292K/HA226Q (inoculum) and viruses isolated from nasal samples of macaques infected with NA292K/HA226Q were inoculated into the MDCK cells. Averages and standard deviations of triplicates are shown. MDCK cells were cultured with viruses for 1 h at an m.o.i. of 0.01, then the viral inoculum was replaced by medium containing oseltamivir acid at a concentration between 0 – 10 $\mu\text{g}/\text{mL}$. Culture supernatants were collected after 24 h for virus titration. Averages and standard deviations of quadruplicates are shown. Asterisks indicate significant differences compared with the virus titers without oseltamivir ($*P < 0.05$, Mann-Whitney U test).

Table 1. Neuraminidase inhibition of viruses with NA substitutions

Virus	IC ₅₀ (nM) (Average +/- SD) ^a	
	oseltamivir	peramivir
rgAnhui/1 (HA226L)	0.150 +/- 0.088	0.043 +/- 0.021
rgNA222T/HA226L	0.956 +/- 0.544	0.163 +/- 0.098
rgNA292K/HA226L	1350.522 +/- 400.051	109.259 +/- 50.292
rgHA226Q	0.242 +/- 0.137	0.084 +/- 0.077
rgNA222T/HA226Q	0.718 +/- 0.631	0.157 +/- 0.085
rgNA292K/ HA226Q	2331.677 +/- 409.293	154.701 +/- 31.965

^a Averages and standard deviations of four experiments.

Table 2. Nucleotide and amino acid changes in NA222 of viruses isolated from nasal samples.

Monkey	Days after virus inoculation	Total no. of coverage ^b	% of nucleotide at 656 ^a —AA at 222		
			C ^c	T ^d	in major population
Inoculum	-	14,151	99.80	0.13	T
T1	3	17,048	99.65	0.25	T
T1	5	20,045	99.12	0.70	T
T1	7	18,391	99.61	0.31	T
T2	3	19,569	99.62	0.30	T
T2	5	21,269	99.50	0.39	T
T2	7	18,021	99.48	0.39	T
T3	3	16,667	99.69	0.22	T
T3	5	16,384	99.25	0.60	T

^a Nucleotides at position 656 in the NA gene corresponding to amino acid position 222 in the NA protein (N2 numbering) were examined. Percentages of the nucleotide alleles were calculated as % alleles = $100 \times (\text{number of alleles counted}/\text{total coverage number})$.

^b Total number of sequences counted at each nucleotide position.

^c A nucleotide at 656 of inoculum virus is C corresponding to threonine (T).

^d A nucleotide at 656 of wild-type virus (Anhui/1) is T corresponding to isoleucine (I).

Table 3. Nucleotide and amino acid changes in NA292 of viruses isolated from nasal samples.

Monkey	Days after virus inoculation	Total no. of coverage ^b	% of nucleotide at 866 ^a —AA at 292		
			A ^c	G ^d	in major population
Inoculum	-	16,354	99.79	0.11	K
K1	3	17,995	86.88	12.87	K
K1	5	15,345	36.57	63.26	R
K1	7	20,780	0.16	99.56	R
K2	5	17,585	16.41	83.32	R
K3	3	14,824	73.00	26.68	K
K3	5	16,874	51.03	48.74	K
K3	7	16,310	26.56	73.30	R

^a Nucleotides at position 866 in the NA gene corresponding to amino acid (AA) position 292 in the NA protein (N2 numbering) were examined. Percentages of the nucleotide alleles were calculated as $100 \times (\text{number of alleles counted}/\text{total coverage number})$.

^b Total number of sequences counted at each nucleotide position.

^c A nucleotide at 866 of inoculum virus is A corresponding to lysine (K).

^d A nucleotide at 866 of wild-type virus (Anhui/1) is G corresponding to arginine (R).

Fig. 1

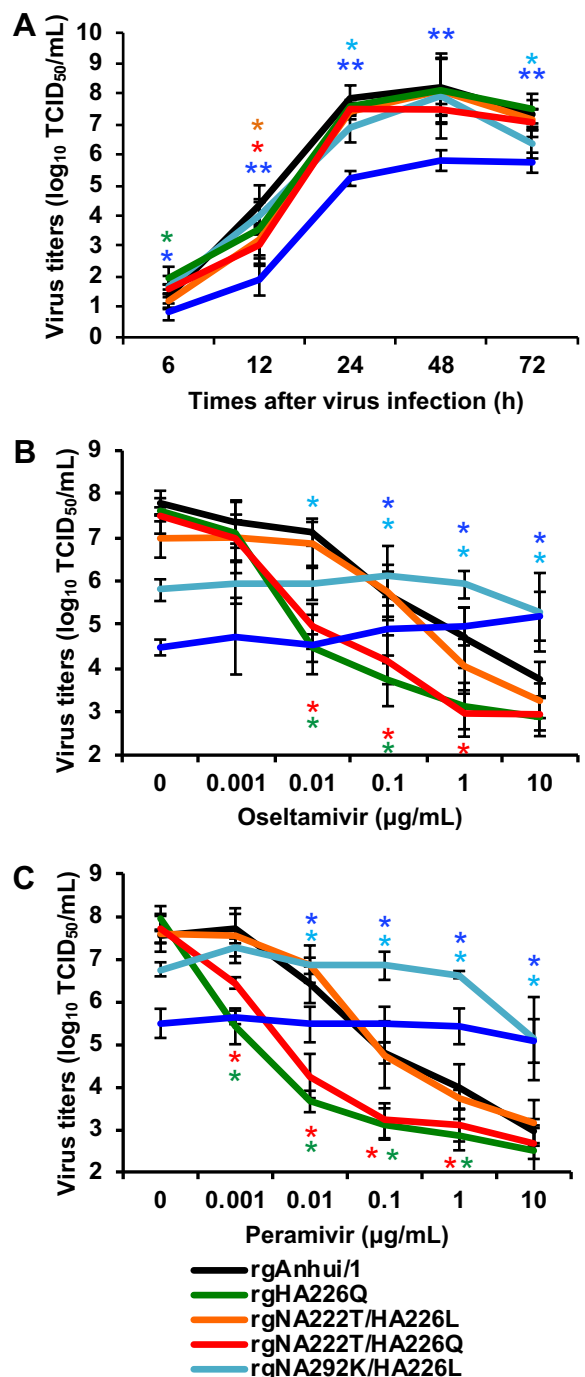


Fig 1. Replication and sensitivity to anti-viral drugs of variant viruses carrying NA222T, NA292K, and HA226Q in MDCK cells.

(A) Variant viruses carrying HA226L, HA226Q, wild-type NA, NA222T, or NA292K were inoculated into culture of MDCK cells at an m.o.i. of 0.01 to determine propagation rates. (B, C) MDCK cells were cultured with viruses for 1 h at an m.o.i. of 0.01, and then the viral inoculum was replaced by a medium containing oseltamivir acid (B) or peramivir hydrate (C), at a concentration between 0 – 10 $\mu\text{g/mL}$. Culture supernatants were collected after 24 h for virus titration. Averages and standard deviations of hexaplicates are shown. Asterisks indicate significant differences compared with rgAnhui/1 (HA226L, wild-type) (* $P < 0.05$, ** $P < 0.01$, Mann-Whitney U test).

Table S1. Clinical scoring used in the present study.

Parameter	Degree of parameter	Possible score
Fever	Normal (< 39 °C)	0
	Elevated temperature (39-40 °C)	3
	High temperature (> 40 °C)	5
Posture	Piloerection of body hair	1
	Decreased activity, decreasing normal behavior /Occasionally lying down, huddled, active when people in room	2
	Huddled on camera, active when people in room/Lying down, getting up when approached, using cage for support	3
	Huddled when people in room, shaking, toes and hands clenched/Lying down, not getting up when approached or prompted	5
Respiration	Increased or decreased; mild cough and clear nasal discharge	3
	Labored breathing through mouth; severe cough and severe nasal discharge	5
Appetite	Slightly decreased	1
	Decreased	2
	Severely decreased	5
Skin	Flushed appearance	2
	Visible rash	2
	Bleeding	5

Animals were monitored every day during the study to be clinically scored. Animals would be scheduled to be euthanized if their clinical scores reached 15 (humane endpoint).

Table S2. Nucleotide variations in the NA and HA gene of inoculum virus of NA222T/HA226Q and NA292K/HA226Q used in the present study.

Nucleotide position ^a	Inoculum virus	Reference ^b	Total no. of coverage ^c	Inoculum ^d	% of nucleotide ^e	AA position ^f	AA in reference	AA in sample
NA								
498	NA222T/HA226Q	C	14,704	G	6.93	170	Y ^g	* ^h
656	NA222T/HA226Q	T	14,151	C	99.80	222	I	T
866	NA292K/HA226Q	G	14,979	A	99.79	292	R	K
HA								
319	NA292K/HA226Q	C	11,872	A	8.24	115	P	T
437	NA292K/HA226Q	C	13,843	A	21.36	154	A	E
704	NA222T/HA226Q	T	17,118	A	99.80	226	L	Q
	NA292K/HA226Q	T	12,122	A	99.68		L	Q

^a Nucleotide position 1 is the first nucleotide A of the ATG-translation start point for methionine.

^b A reference sequence of A/Anhui/1/2013 (H7N9) obtained from the GISAID database.

^c Total number of sequences counted at each nucleotide position.

^d Nucleotides of inoculum viruses for which allele frequencies are higher than 5.0 % and different from those in the reference sequence are shown.

^e Percentages of alleles different from the reference sequence was calculated as % alleles = $100 \times (\text{number of alleles counted}/\text{total coverage number})$.

^f Amino acid (AA) positions were determined on the basis of the N2 neuraminidase and H3 hemagglutinin sequences.

^g Amino acid abbreviations. A: alanine, D: aspartic acid, E: glutamic acid, L: leucine, N: asparagine, P: proline, S: serine, T: threonine, Y: tyrosine.

^h * stop codon

Fig. S1

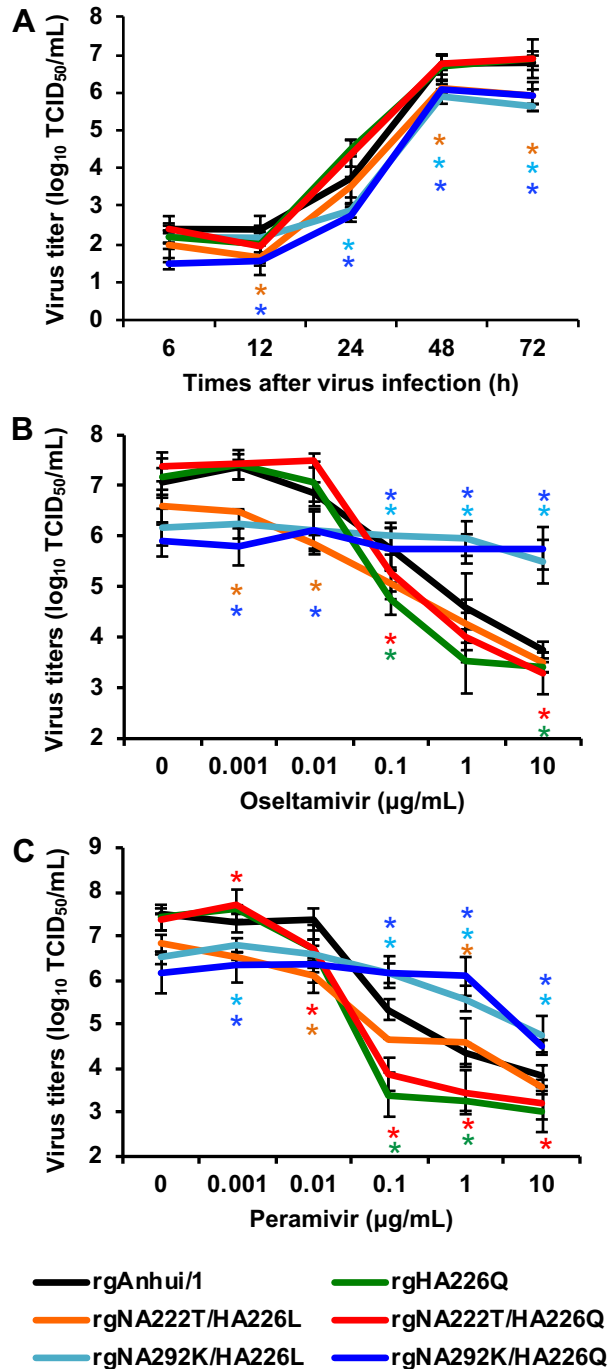


Fig. S1. Replication and sensitivity to anti-viral drugs of variant viruses A549 cells.

(A) Variant viruses carrying wild type HA226L, HA226Q, wild type NA, NA222T, or NA292K were inoculated into culture of A549 cells at an m.o.i. of 0.01 to determine propagation rates. (B, C) A549 cells were cultured with viruses for 1 h at an m.o.i. of 0.01, and then the viral inoculum was replaced by a medium containing oseltamivir acid (B) or peramivir hydrate (C) at a concentration between 0 – 10 $\mu\text{g/mL}$. Culture supernatants were collected after 48 h for virus titration. Averages and standard deviations of triplicates are shown. Asterisks indicate significant differences compared with rgAnhui/1 (HA226L, wild type) (* $P < 0.05$, ** $P < 0.01$, Mann-Whitney U test).

Table S3. EC₅₀ values of NAIs

Virus	EC ₅₀ (µg/mL) ^a			
	MDCK		A549	
	oseltamivir	peramivir	oseltamivir	peramivir
rgAnhui/1	0.102	0.058	0.142	0.082
rgNA222T/HA226L	0.218	0.059	0.095	0.062
rgNA292K/HA226L	1.118	1.594	0.619	0.398
rgHA226Q	0.006	0.002	0.060	0.024
rgNA222T/HA226Q	0.011	0.004	0.109	0.026
rgNA292K/HA226Q	> 10	> 10	> 10	1.716

^a EC₅₀ values of oseltamivir and peramivir were calculated on the basis of the results in Fig. 1 and Fig. S1.

Fig. 2

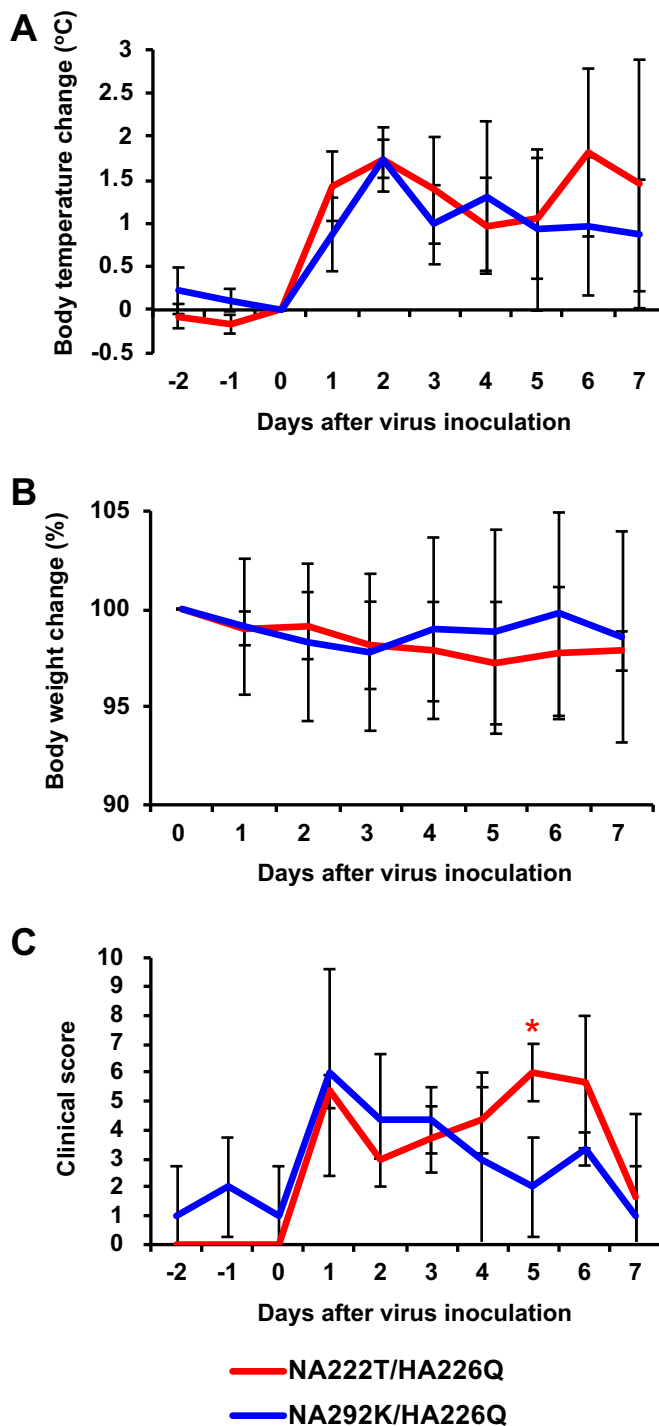


Fig 2. Clinical symptoms of macaques infected with NA222T/HA226Q and NA292K/HA226Q viruses. Cynomolgus macaques ($n = 3$ in each group) were inoculated with influenza viruses NA222T/HA226Q or NA292K/HA226Q that were NAI-resistant variants originated from A/Anhui/1/2013 (H7N9). (A) Average temperature from 8 p.m. to 8 a.m. on the next day were calculated on the basis of data for individual macaques (Fig. S1). Average temperatures on each day were compared with those from 8 p.m. on the day before virus inoculation (day -1) to 8 a.m. on the day of virus inoculation (day 0). (B) Body weights on each day were compared with that on day 0 before virus inoculation. (C) Clinical scores were determined by daily observation and body temperature according to Table S1. Average and standard deviations of the results of three monkeys are shown. Red: macaques inoculated NA222T/HA226Q, blue: macaques inoculated NA292K/HA226Q. An asterisk indicates significant differences between two groups on day 5 ($P < 0.05$, Mann-Whitney U test).

Fig. 2

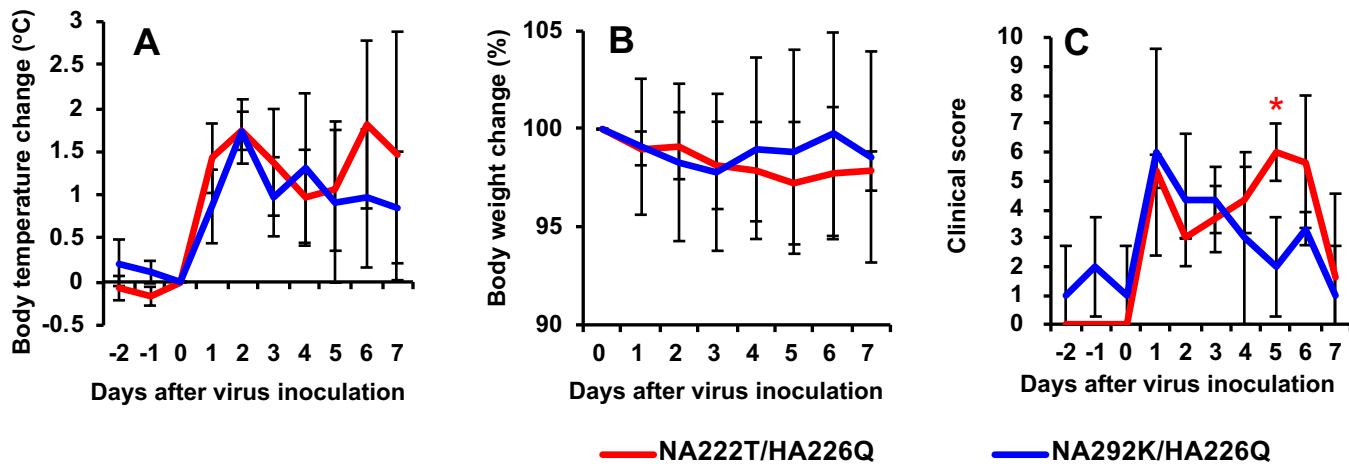


Fig 2. Clinical symptoms of macaques infected with NA222T/HA226Q and NA292K/HA226Q viruses.

Cynomolgus macaques ($n = 3$ in each group) were inoculated with influenza viruses NA222T/HA226Q or NA292K/HA226Q that were NAI-resistant variants originated from A/Anhui/1/2013 (H7N9). (A) Average temperature from 8 p.m. to 8 a.m. on the next day were calculated on the basis of data for individual macaques (Fig. S1). Average temperatures on each day were compared with those from 8 p.m. on the day before virus inoculation (day -1) to 8 a.m. on the day of virus inoculation (day 0). (B) Body weights on each day were compared with that on day 0 before virus inoculation. (C) Clinical scores were determined by daily observation and body temperature according to Table S1. Average and standard deviations of the results of three monkeys are shown. Red: macaques inoculated NA222T/HA226Q, blue: macaques inoculated NA292K/HA226Q. An asterisk indicates significant differences between two groups on day 5 ($P < 0.05$, Mann-Whitney U test).

Fig. S2

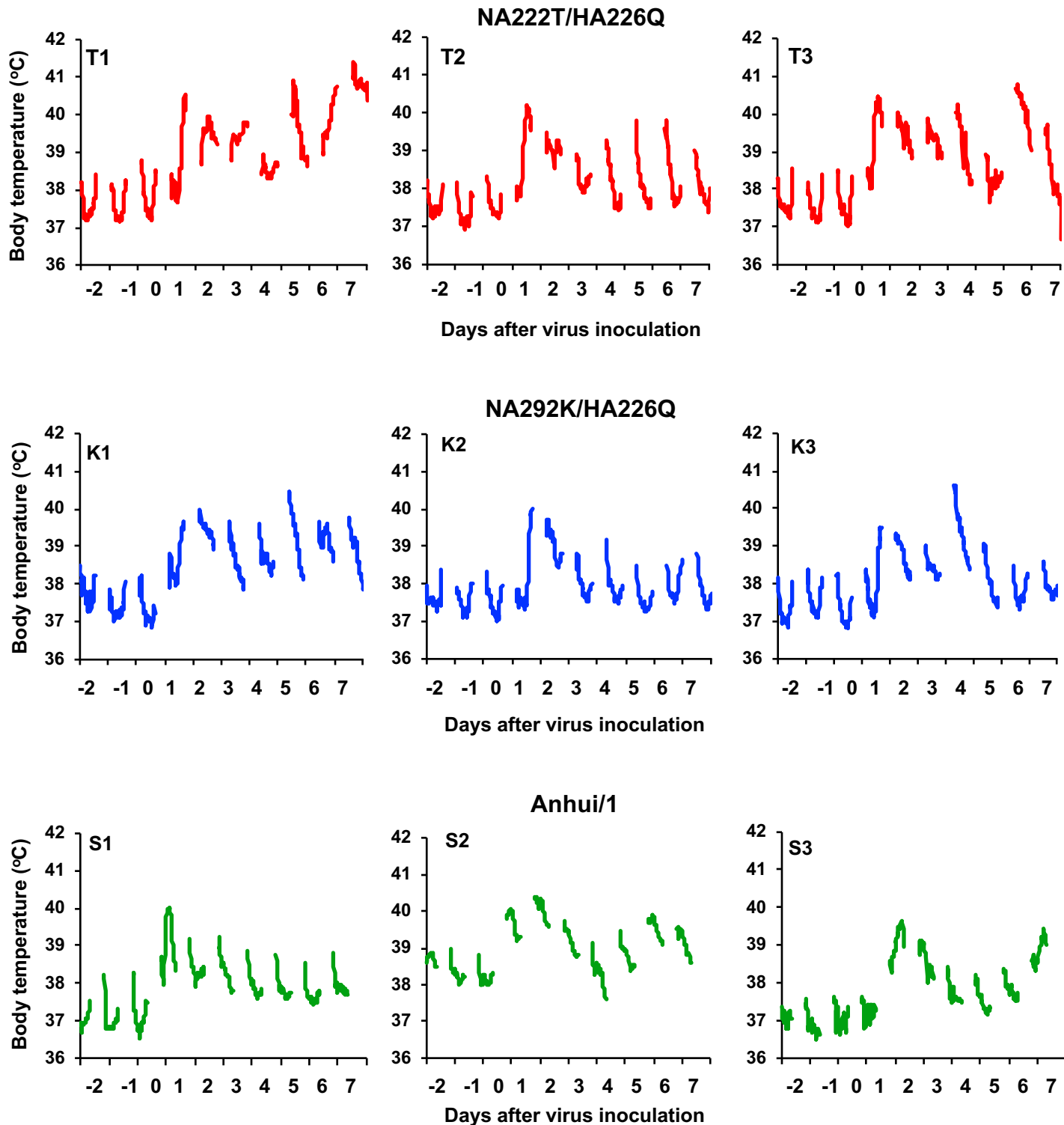


Fig. S2. Body temperatures of macaques infected with NA222T/HA226Q and NA292K/HA226Q viruses. Cynomolgus macaques ($n = 3$ in each group) were inoculated NAIs resistant influenza viruses, NA222T/HA226Q (red), NA292K/HA226Q (blue), or Anhui/1 (green). Body temperatures of the macaques were recorded using telemetry transmitters and a computer. Temperatures from 8 P.M. to 8 A.M. are shown in the graphs since temperatures between 8 A.M. to 8 P.M. were affected by anesthesia. Results of macaques infected with Anhui/1 were cited from our previous study (Itoh, et al., 2015) and the graphs were remade.

Fig. 3

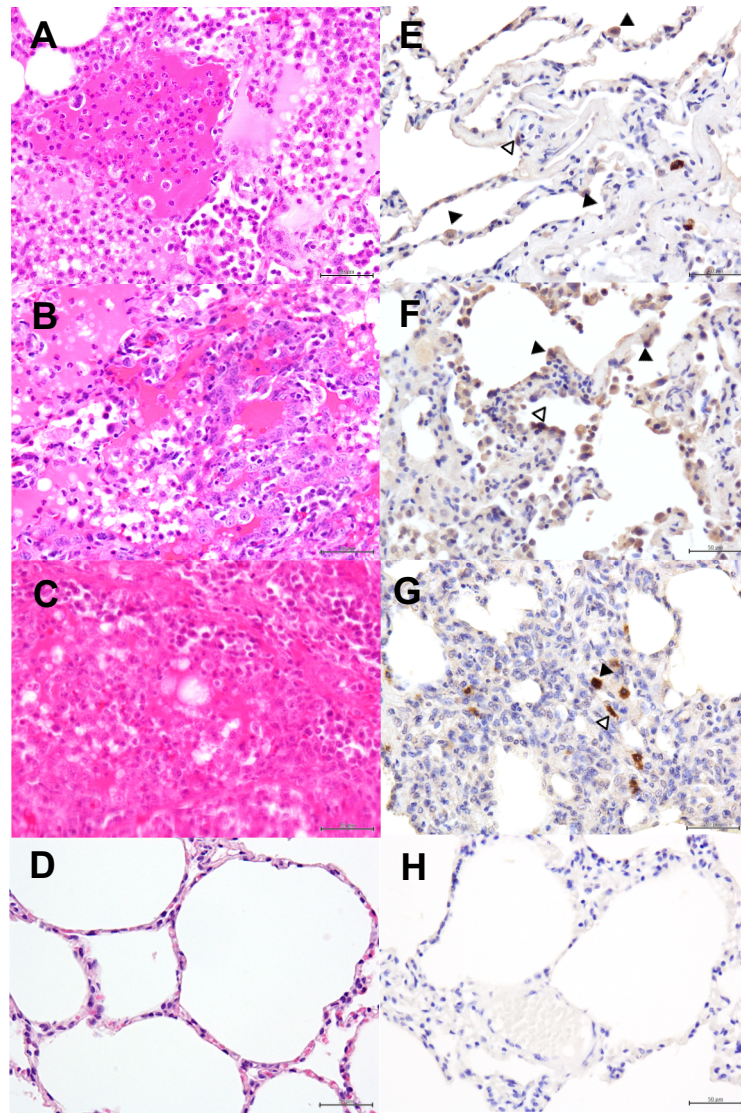


Fig 3. Viral pneumonia in macaques infected with NA222T/HA226Q and NA292K /HA226Q viruses. All macaques infected with virus were autopsied 7 days after virus inoculation. Representative photos for each group are shown. (A, E) Lung tissues of macaques infected with NA222T/HA226Q (T1). (B, F) Lung tissues of macaques infected with NA292K/HA226Q (K2). (C, G) Lung tissues of macaques infected with Anhui/1 in our previous study (Itoh et al., 2015). Sections were newly prepared and stained. (D, H) Lung tissues of a normal uninfected macaque. (A-D) H&E staining. (E-H) and immunohistochemical staining for influenza virus NP (brown). White arrowheads: type-I alveolar epithelial cells, black arrowheads: type-II alveolar epithelial cells, bars: 50 μ m.

Fig. 4

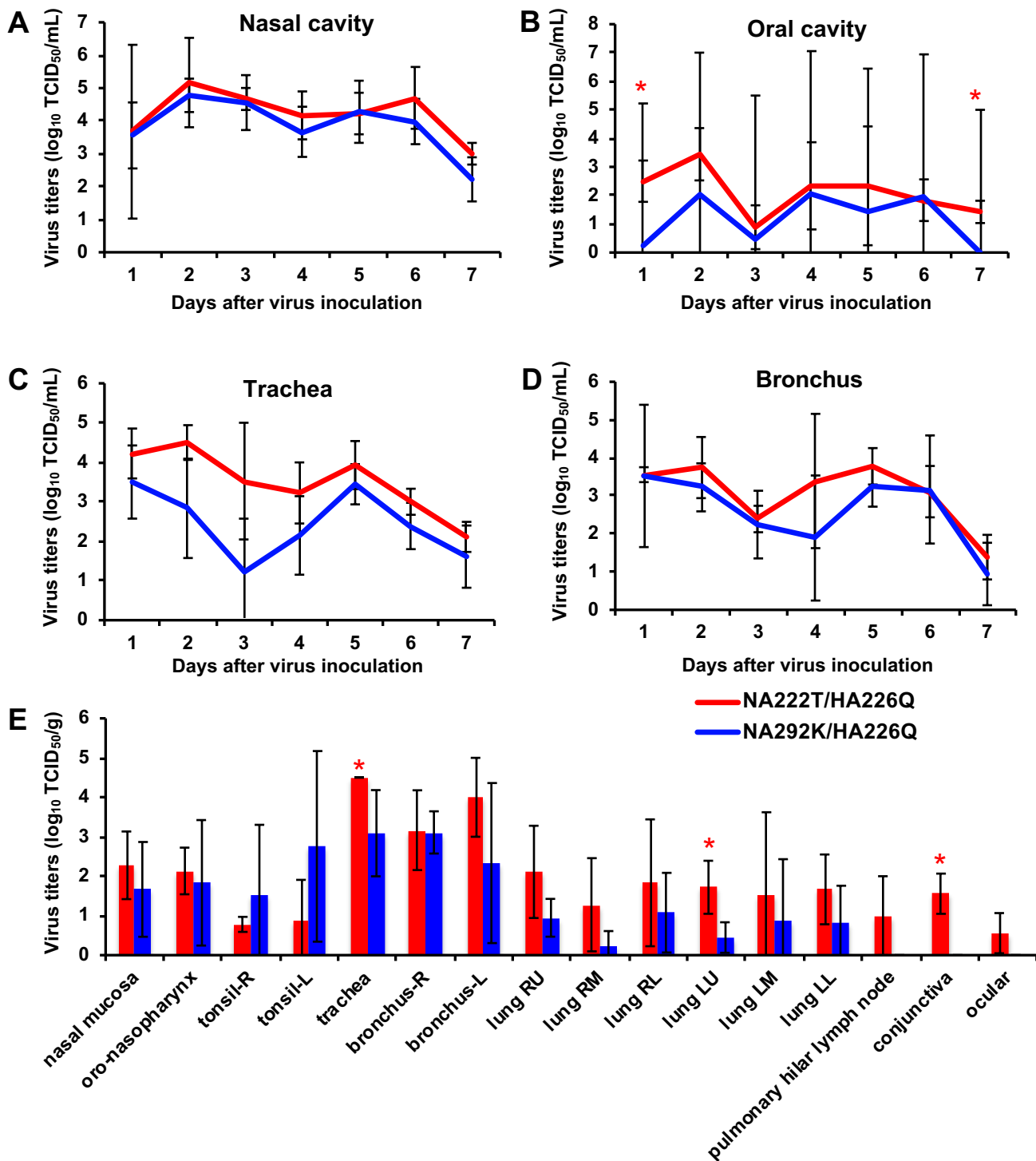


Fig 4. Virus titers in swab and tissue samples from macaque infected with NA222T/HA226Q and NA292K /HA226Q viruses.

Cynomolgus macaques were inoculated with NA222T/HA226Q and NA292K /HA226Q on day 0. (A-D) Nasal, oral, tracheal, and bronchial samples were collected on the indicated days. Averages and standard deviations of virus titers in the nasal (A), oral (B), tracheal (C), and bronchial (D) samples were calculated on the basis of individual titers listed in Table S2. (E) Averages of virus titers in tissue samples. The samples were collected at autopsy 7 days after virus inoculation. R: right, L: left, RU: right upper lobe, RM: right middle lobe, RL: right lower lobe, LU: left upper lobe, LM: left middle lobe, LL: left lower lobe. Virus titers under the detection limit were calculated as 0. Averages and standard deviations of the results for three macaques are shown. Red: macaques inoculated with NA222T/HA226Q virus, blue: macaques inoculated with NA292K/HA226Q virus. Asterisks indicate significant differences between the two groups ($P < 0.05$, Mann-Whitney U test).

Table S4. Virus titers in swab samples of cynomolgus macaques infected with NA222T/HA226Q and NA292K/HA226Q.

Sample	Virus	Animals	Virus titers (\log_{10} TCID ₅₀ /mL)						
			Days after virus inoculation						
			1	2	3	4	5	6	7
Nasal swab	NA222T/HA226Q	T1	5.67	6.33	5.00	4.50	4.67	4.33	3.33
		T2	≤ 0.67 ^a	3.67	4.67	3.33	3.50	5.77	3.00
		T3	4.67	5.50	4.33	4.67	4.50	4.00	2.67
	NA292K/HA226Q	K1	3.67	4.33	4.67	4.50	4.00	3.50	1.50
		K2	4.50	4.67	3.67	3.00	5.33	4.77	2.33
		K3	2.50	5.33	5.33	3.50	3.50	3.67	2.83
	Anhui/1 ^b	S1	5.00	4.00	4.00	1.67	2.50	3.67	1.83
		S2	4.33	3.00	2.67	3.50	4.77	3.67	< 1.67 ^a
		S3	5.33	5.23	3.50	3.00	4.00	4.67	2.33
Oral swab	NA222T/HA226Q	T1	2.83	3.50	≤ 1.33 ^a	2.67	4.67	2.67	≤ 1.50 ^a
		T2	1.67	2.50	≤ 1.33 ^a	≤ 0.67 ^a	≤ 0.67 ^a	1.50	≤ 1.77 ^a
		T3	3.00	4.33	< ^c	3.67	1.67	1.33 ^a	≤ 1.00 ^a
	NA292K/HA226Q	K1	<	1.67	1.50	2.00	2.00	2.00	<
		K2	<	1.67	<	3.50	2.33	≤ 1.33 ^a	<
		K3	≤ 0.67 ^a	2.67	<	≤ 0.67 ^a	<	2.50	<
	Anhui/1	S1	2.67	0.67	2.17	<	2.33	1.00	< 1.67
		S2	<	1.67	<	<	0.67	0.67	<
		S3	1.00	3.33	0.83	2.00	3.50	<	<
Tracheal swab	NA222T/HA226Q	T1	4.50	4.67	5.23	3.67	4.33	3.33	2.33
		T2	3.50	4.00	2.67	2.33	3.23	2.67	2.33
		T3	4.67	4.83	2.67	3.67	4.23	3.00	1.67
	NA292K/HA226Q	K1	4.50	3.00	2.67	2.77	3.33	3.00	2.50
		K2	2.67	1.50	<	2.67	4.00	1.83	≤ 1.33 ^a
		K3	3.33	4.00	≤ 1.00 ^a	≤ 1.00 ^a	3.00	2.33	≤ 1.00 ^a
	Anhui/1	S1	6.33	5.00	3.00	1.00	3.00	3.77	<
		S2	3.50	1.50	2.50	1.33	4.77	3.50	<
		S3	4.77	4.00	3.00	3.00	4.00	3.00	< 1.67
Bronchial swab	NA222T/HA226Q	T1	3.67	4.67	2.67	5.00	4.33	2.33	2.00
		T2	3.33	3.23	2.00	1.50	3.50	3.50	≤ 0.83 ^a
		T3	3.67	3.33	2.50	3.67	3.50	3.50	≤ 1.33 ^a
	NA292K/HA226Q	K1	5.67	3.50	3.23	2.67	3.67	4.50	≤ 1.33 ^a
		K2	2.23	2.50	1.50	3.00	3.33	3.33	1.50
		K3	2.67	3.67	≤ 2.00 ^a	<	2.67	1.67	<
	Anhui/1	S1	3.00	4.50	1.50	2.67	1.50	1.50	<
		S2	3.00	0.67	<	2.50	1.67	<	<
		S3	3.50	3.00	1.33	1.50	2.00	2.33	<

^a A part of four wells were positive for CPE in quadruplicate culture of undiluted samples.

^b Virus titers in samples of macaques infected with Anhui/1 are cited from our previous study (Itoh, et al, 2015) for comparison.

^c <: virus titers were under the detection limit (0.67 \log_{10} TCID₅₀/mL).

Fig. S3.

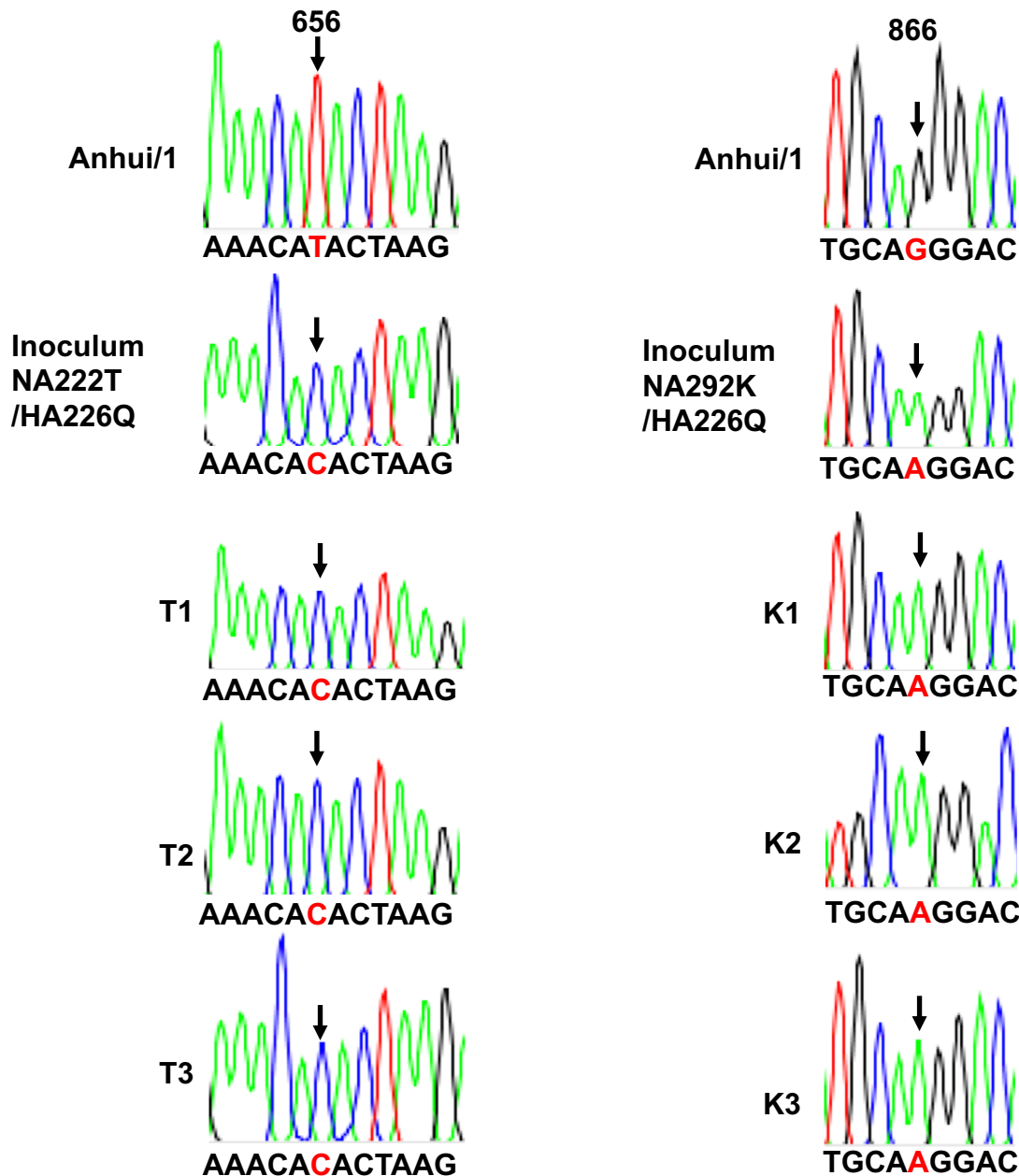


Fig. S3. NA gene sequences of the viruses in lung tissues of macaques infected with NA222T/HA226Q and NA292K/HA226Q viruses.

RNA was extracted from the lung tissues of macaques infected with NA222T/HA226Q virus (T1 - T3) or NA292K/HA226Q virus (K1 - K3) 7 days after infection. Extracted RNA was reverse-transcribed and partial NA genes were amplified with KOD plus-neo DNA polymerase. The used primers were 5'-TGCACCTCAGCCACTGCTAT-3' and 5'-ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT-3' in the first PCR and 5'-GTGGAATGCATTGGGTGGTC-3' 5'-ATATCGTCTCGTATTAGTAGAAACAAGGAGTTTTTT-3' in the second PCR. After denaturation at 94 °C for 2 min, the reaction was performed with 35 (1st PCR) and 30 (2nd PCR) cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 68 °C for 45 s, followed by extension at 68 °C for 5 min. Nucleotide sequences were determined by Sanger sequencing. Nucleotide sequences of Anhui/1 and inoculum viruses are shown for comparison.

Table S5. Nucleotide and amino acid changes in NA of NA222T/HA226Q and NA292K/HA226Q.

Virus ^a	Lot	Passage	nucleotide position; 656			AA position	nucleotide position; 866			AA position
			Total no. of coverage ^b	% of nucleotide ^c		AA of major population ^d	Total no. of coverage ^b	% of nucleotide ^c		AA of major population ^d
				C	T			A	G	
NA222T /HA226Q	1	1	21,912	99.69	0.22	T	23,579	0.05	99.87	R
		2	22,933	99.73	0.20	T	24,062	0.05	99.85	R
		3	20,582	99.73	0.20	T	21,984	0.05	99.82	R
	2	1	21,523	99.75	0.18	T	22,387	0.06	99.81	R
		2	21,737	99.77	0.14	T	22,355	0.04	99.86	R
		3	21,437	99.73	0.22	T	23,034	0.08	99.81	R
	3	1	16,544	99.69	0.23	T	17,525	0.03	99.88	R
		2	22,040	99.77	0.18	T	21,816	0.05	99.84	R
		3	16,842	99.73	0.22	T	18,613	0.12	99.78	R
NA292K /HA226Q	1	1	22,589	0.11	99.81	I	20,640	99.65	0.23	K
		2	25,437	0.14	99.70	I	24,502	99.67	0.24	K
		3	28,177	0.12	99.80	I	25,149	99.59	0.28	K
	2	1	24,749	0.06	99.88	I	22,743	99.68	0.23	K
		2	26,582	0.08	99.83	I	24,017	99.65	0.24	K
		3	26,773	0.12	99.76	I	24,524	99.64	0.18	K
	3	1	24,910	0.11	99.77	I	22,539	99.68	0.23	K
		2	21,176	0.08	99.82	I	23,153	99.62	0.25	K
		3	23,371	0.09	99.81	I	21,158	99.56	0.30	K

^a Viruses passaged in MDCK are used from same stock lot as that used in inoculation of macaques on day 0.

^b Total number of sequences counted at each nucleotide position

^c Percentage of alleles of the indicated nucleotide was calculated as % alleles = 100 × (number of alleles counted/total coverage number).

^d Amino acids (AA) coded by major nucleotides counted at the position. AA positions are determined on the basis of an N2 numbering. I: isoleucine, K: lysine, R: arginine, T: threonine.

Table S6. Allele frequency in virus in nasal samples collected from macaques infected with NA292K/HA226Q virus after a passage in MDCK cells.

Monkey ^a	Days after virus inoculation ^b	Total no. of coverage	% nucleotide at position 866		AA of majority population at NA292
			A	G	
K1	1	22,610	99.68	0.23	K
K1	7	20,223	0.11	99.72	R
K3	1	24,598	99.47	0.42	K
K3	7	21,727	13.58	86.36	R

^a Monkeys K1 and K3 were inoculated with NA292K/HA226Q virus on day 0.

^b Nasal swab samples were collected on days 1 and 7. The samples were inoculated to MDCK cells to make stock viruses. Then, RNA was extracted for deep sequencing.

Fig. 5

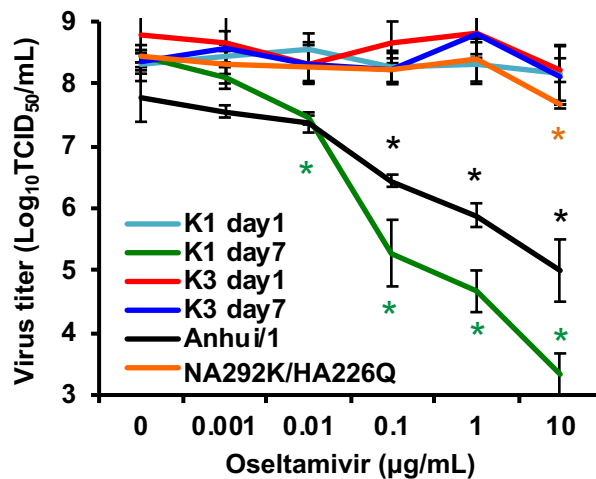


Fig 5. Viral sensitivity to oseltamivir of viruses isolated from nasal samples in MDCK cells.

Anhui/1 (wild-type), NA292K/HA226Q (inoculum) and viruses isolated from nasal samples of macaques infected with NA292K/HA226Q on indicated days were inoculated into the MDCK cells. Averages and standard deviations of triplicates are shown. MDCK cells were cultured with viruses for 1 h at an m.o.i. of 0.01, then the viral inoculum was replaced by medium containing oseltamivir acid at the concentration between 0 – 10 µg/mL. Culture supernatants were collected after 24 h for virus titration. Averages and standard deviations of quadruplicates are shown. Asterisks indicate significant differences compared with the virus titers without oseltamivir ($*P < 0.05$, Mann-Whitney U test).