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Substitutions T200A and E227A in the Hemagglutinin of Pandemic 2009 Influenza A Virus Increase Lethality but Decrease Transmission

Carles Martínez-Romero,[a,c] Erik de Vries,[e] Alan Belicha-Villanueva,[a,c] Ignacio Mena,[a,c] Donna M. Tscherne,[a,c] Virginia L. Gillespie,[d] Randy A. Albrecht,[a,c] Cornelis A. M. de Haan,[e] Adolfo García-Sastre[a,b,c]

Department of Microbiology,[a] Department of Medicine, Division of Infectious Diseases,[b] and Global Health and Emerging Pathogens Institute,[c] Icahn School of Medicine at Mount Sinai, New York, New York, USA; Center for Comparative Medicine and Surgery, Comparative Pathology Laboratory, Icahn School of Medicine at Mount Sinai, New York, New York, USA[d]; Virology Division, Department of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands[e]

We report that swine influenza virus-like substitutions T200A and E227A in the hemagglutinin (HA) of the 2009 pandemic influenza virus alter its pathogenesis and transmission. Viral replication is increased in mammalian cells. Infected mice show increased disease as measured by weight loss and lethality. Transmission in ferrets is decreased in the presence of both substitutions, suggesting that amino acids 200T and 227E are adaptive changes in the HA of swine origin influenza viruses associated with increased transmission and decreased pathogenesis.

Modifications in the hemagglutinin (HA) glycoprotein of the influenza A virus are believed to be a catalyst for previous world pandemics (1, 2). This includes reassortment between cocirculating animal and human viruses and mutations in the HA which grant better transmissibility between hosts (3). The pandemic influenza A virus from 2009, A(H1N1)pdm09, originated from reassortment among three cocirculating swine and avian-like viruses; hence, its HA comes from a swine virus origin (4). Although little is known about the specific requirements for the swine influenza virus to adopt human-to-human transmissibility, minimal adaptation in the HA sequence might suffice (5).

Different residues within and near the receptor-binding site of the HA in A(H1N1)pdm09 viruses have been found to specifically alter receptor affinity. Residues 200 and 227 were recently reported to be responsible for the difference in the binding affinity for the sialic acid receptors between the HAs of swine and A(H1N1)pdm09 influenza virus isolates (6). Furthermore, recent publications have shed light on the role of HA residue 227 in cell tropism and transmission, confirming its role in sialic acid recognition (7). In swine influenza virus isolates, amino acids 200 and 227 of the HA are alanines (A), while in some A(H1N1)pdm09 viruses, residue 200 is a threonine (T) and 227 is invariably a glutamic acid (E). In fetuin binding and glycan array assays, it was observed that swine virus-derived HA showed stronger binding to α2,6-linked sialic acids. When substitutions T200A and E227A were introduced into A(H1N1)pdm09 HA, its receptor binding increased to the same level as its swine HA counterpart (6).

We sought to characterize the effect of these two substitutions in viral replication, pathogenicity, and transmission. Using plasmid-based reverse genetics (8), we generated recombinant influenza viruses with HA mutations T200A and E227A by using the A/California/04/2009 strain as the backbone. A wild-type (WT) recombinant (rCal/09 WT) was generated, as well as three HA substitution mutants, one bearing T200A (rCal/09 HA T200A), another with E227A (rCal/09 HA E227A), and a third containing both substitutions (rCal/09 HA T200A/E227A). All recombinant viruses were plaque purified at least two times, and full-genome sequencing of the viruses was conducted to confirm the presence of both substitutions, as well as the absence of any other, undesired, mutation. We determined the hemagglutination titer of our recombinant virus stocks (10^6 PFU for each virus) in 1% turkey red blood cells (Fig. 1). Viruses with T200A and/or E227A showed a clear increase in hemagglutination activity compared to that of rCal/09 WT. We

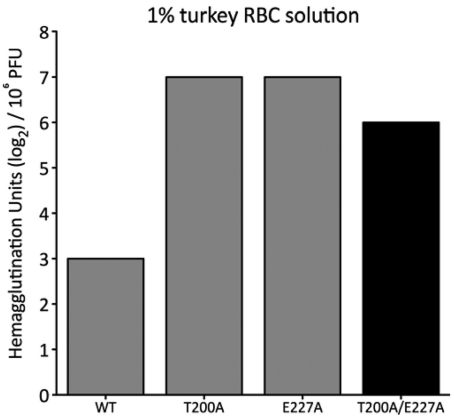

FIG 1 Substitutions T200A and E227A in the HA of Cal/09 increase hemagglutination activity. Viruses were normalized to 10^6 PFU, and then 2-fold dilutions were mixed with a 1% turkey red blood cell solution in phosphate-buffered saline. Hemagglutination activity was measured after 45 min of incubation at 4°C.

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Address correspondence to Adolfo García-Sastre, Adolfo.Garcia-Sastre@mssm.edu.

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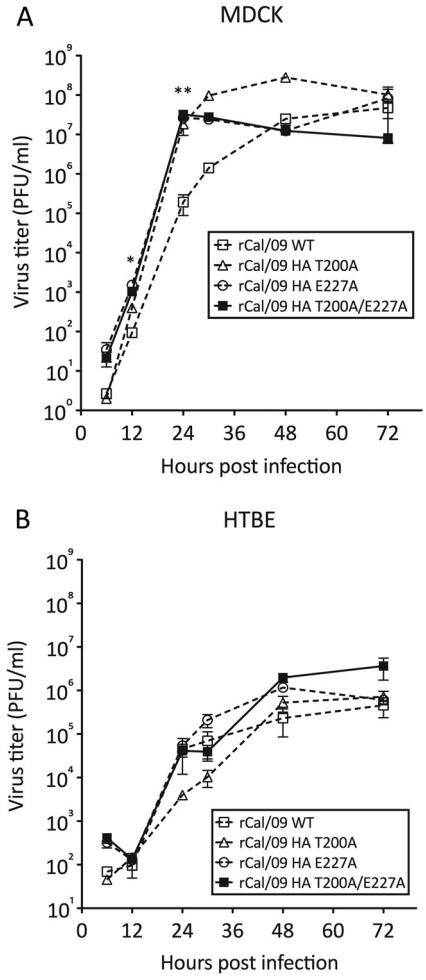

FIG 2 Effects of substitutions T200A and E227A on viral replication in different mammalian cell models. (A) MDCK cells were infected with virus at an MOI of 0.0005 PFU/cell. *, $P < 0.05$; **, $P < 0.01$; error bars depict the standard error of the mean. (B) Eight-week-old cultures of differentiated HTBE cells were infected with each virus at an MOI of 0.1 PFU/cell.

concluded that this might be due to an increase in the affinity of the HA for sialic acids. However, mutant viruses might be producing more noninfectious particles than rCal/09 WT, thus increasing their hemagglutination activity.

In order to characterize alterations in viral replication in mammalian cell models, MDCK cells were infected at a multiplicity of infection (MOI) of 0.0005 PFU/cell (Fig. 2A). All of the mutant viruses displayed higher virus titers than rCal/09 at early time points, with a 2-log difference at 24 h postinfection. Primary human tracheobronchial epithelial (HTBE) cells (Lonza) were also used to test the replication of our recombinant viruses (Fig. 2B). Cells were grown and differentiated for 8 weeks at an air-liquid interface as described previously (9), and then they were used for all experiments. At an MOI of 0.1 PFU/cell, no significant differences between the replication phenotypes of the viruses were found. We concluded that the presence of substitutions T200A and E227A correlate with faster replication of the virus in MDCK cells at early time points, probably because the virus entered these cells more efficiently than rCal/09 WT did. The fact that no difference was found in HTBE cells might be explained by differences in their sialic acid composition from that of MDCK cells.

To further investigate the potential effects of substitutions T200A and E227A on pathogenicity, we characterized them in the context of the DBA/2J mouse model. Groups of eight 8-week-old females were inoculated intranasally with 1,000 PFU of virus and then monitored for weight loss (Fig. 3A) and survival (Fig. 3B) daily for 11 days to assess the effects of the infection. Animals with a body weight loss of more than 25% were considered to have reached the experimental endpoint and were humanely euthanized. Mice infected with rCal/09 WT had a survival rate of 72%. Of the animals infected with the mutated viruses, 100% succumbed to the infection; the group infected with the rCal/09 HA T200A/E227A virus exhibited the most significant morbidity and mortality. In a subsequent experiment, mice infected with the same amount of the respective viruses were euthanized at the indicated time points and viral titers in the lungs were determined by plaque assay (Fig. 3C). As expected, mice infected with rCal/09 HA T200A/E227A displayed higher titers at early time points. We also analyzed the cytokine response and inflammation associated with rCal/09 HA T200A/E227A and rCal/09 WT (Fig. 4). Total RNA was isolated from mouse lungs at 1, 2, and 4 days postinfection, and mRNA levels of beta interferon (IFN-β) (Fig. 4A), tumor necrosis factor alpha (TNF-α) (Fig. 4B), and chemokine (C-X-C motif) ligand 2 (CXCL2) (Fig. 4C) were quantified. Mice infected with the double mutant virus showed an increase in the expression of all three genes, leading to the conclusion that the presence of T200A and E227A leads to a stronger innate immune response and inflammation.

Histopathological analysis of the lungs of mice infected with rCal/09 WT and rCal/09 HA T200A/E227A was performed (Fig. 5). Lung samples were collected at different time points after infection. Lesion scoring was performed in a blind fashion, and all samples were analyzed by the same person. While interstitial damage was similar (data not shown), important differences in bronchiolar necrosis were found. Mice infected with rCal/09 WT showed a peak in peribronchiolar inflammation (Fig. 5A), epithelial degeneration (Fig. 5B), and intraluminal debris (Fig. 5C) at 24 h postinfection that gradually disappeared, correlating with the weight recovery and survival shown above. On the other hand, animals infected with the mutant virus did not show recovery in the affected regions of the lungs, leading to an increase in the aforementioned lesions at 96 h postinfection. We concluded that the presence of substitutions T200A and E227A exacerbates bronchiolar necrosis in mouse lungs, correlating with the increase in the inflammatory and immune response.

Ferrets were used to ascertain whether viral transmission is affected by the presence of substitutions T200A and E227A (Fig. 6). Two animals were infected intranasally with 10^6 PFU of rCal/09 WT, and another two were infected intranasally with 10^6 PFU of rCal/09 HA T200A/E227A. Animals were placed in individual cages, and 1 day after inoculation, naive animals were introduced into the cage, which had a division between the two animals that allowed aerosol transmission exclusively (10). Nasal washes were collected, and viral titers were quantified by plaque assay. Ferrets with rCal/09 WT by aerosol showed a normal transmission pattern (10), peaking 2 days later than the donors and gradually shedding until day 10

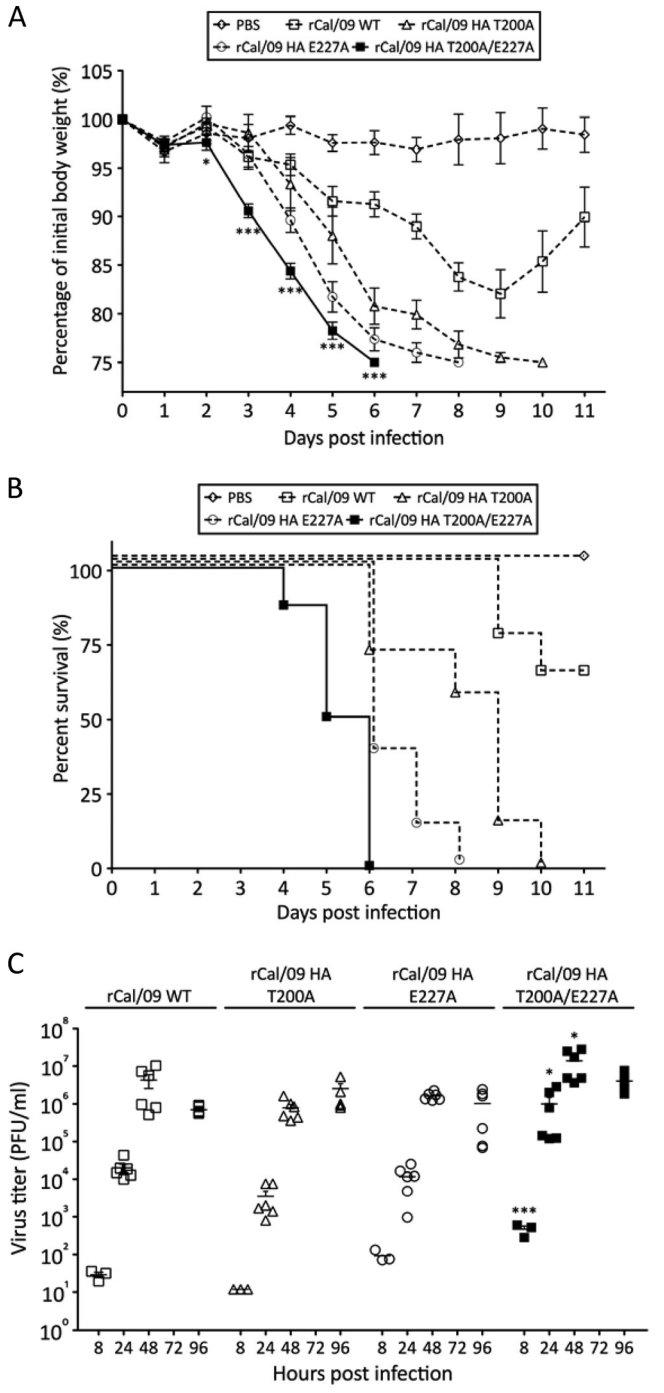

FIG 3 Substitutions T200A and E227A in the HA increase weight loss, lethality, and Cal/09 viral replication in DBA/2J mice. Groups of eight mice were infected intranasally, and weight loss (A) and the survival rate (B) were monitored. (C) Lung tissue was collected from the mice ($n = 6$) at different time points after infection and homogenized, and viral titers were determined by plaque assay. *, $P < 0.05$; ***, $P < 0.001$; error bars depict the standard error of the mean.

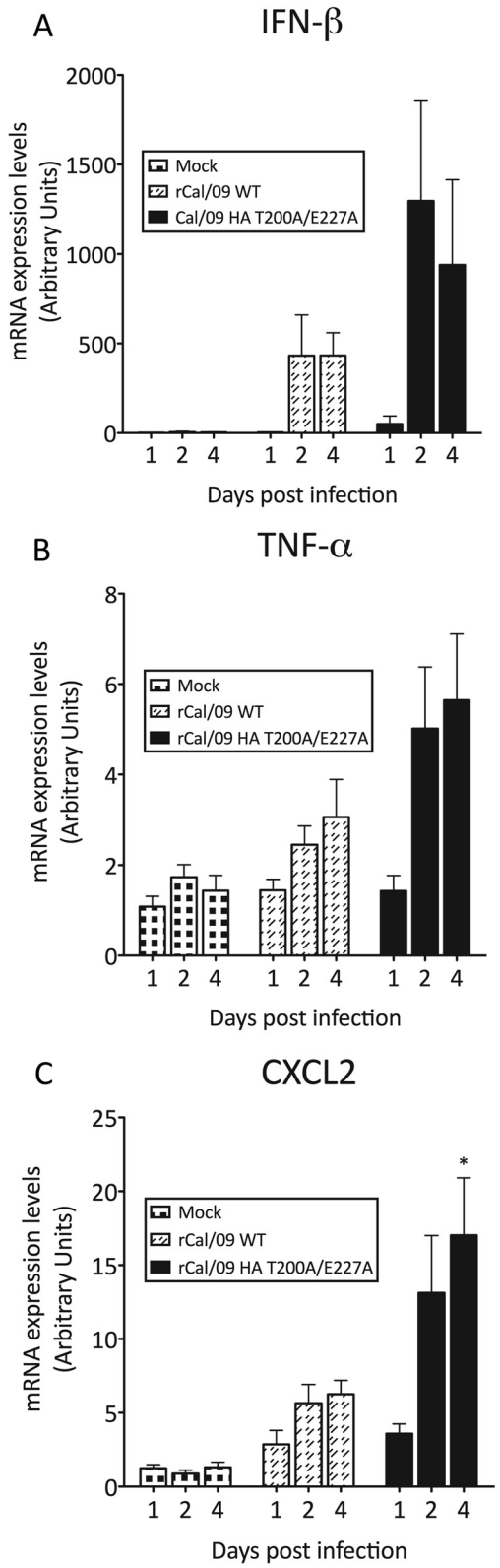

FIG 4 The proinflammatory cytokine response is increased in mice ($n = 6$) infected with rCal/09 HA T200A/E227A. Lung tissues were collected at 1, 2, and 4 days postinfection, and total RNA was isolated. IFN-β (A), TNF-α (B), and CXCL2 (C) mRNA expression levels were quantified by real-time PCR with β-actin levels as a housekeeping control. **, $P < 0.005$; error bars depict the standard error of the mean. PBS, phosphate-buffered saline.

postinfection (Fig. 6A). On the other hand, transmission of rCal/09 HA T200A/E227A was delayed up to 2 days compared to the peak in the titer of the WT virus (Fig. 6B). Even though no weight loss trend was found (data not shown), pathological

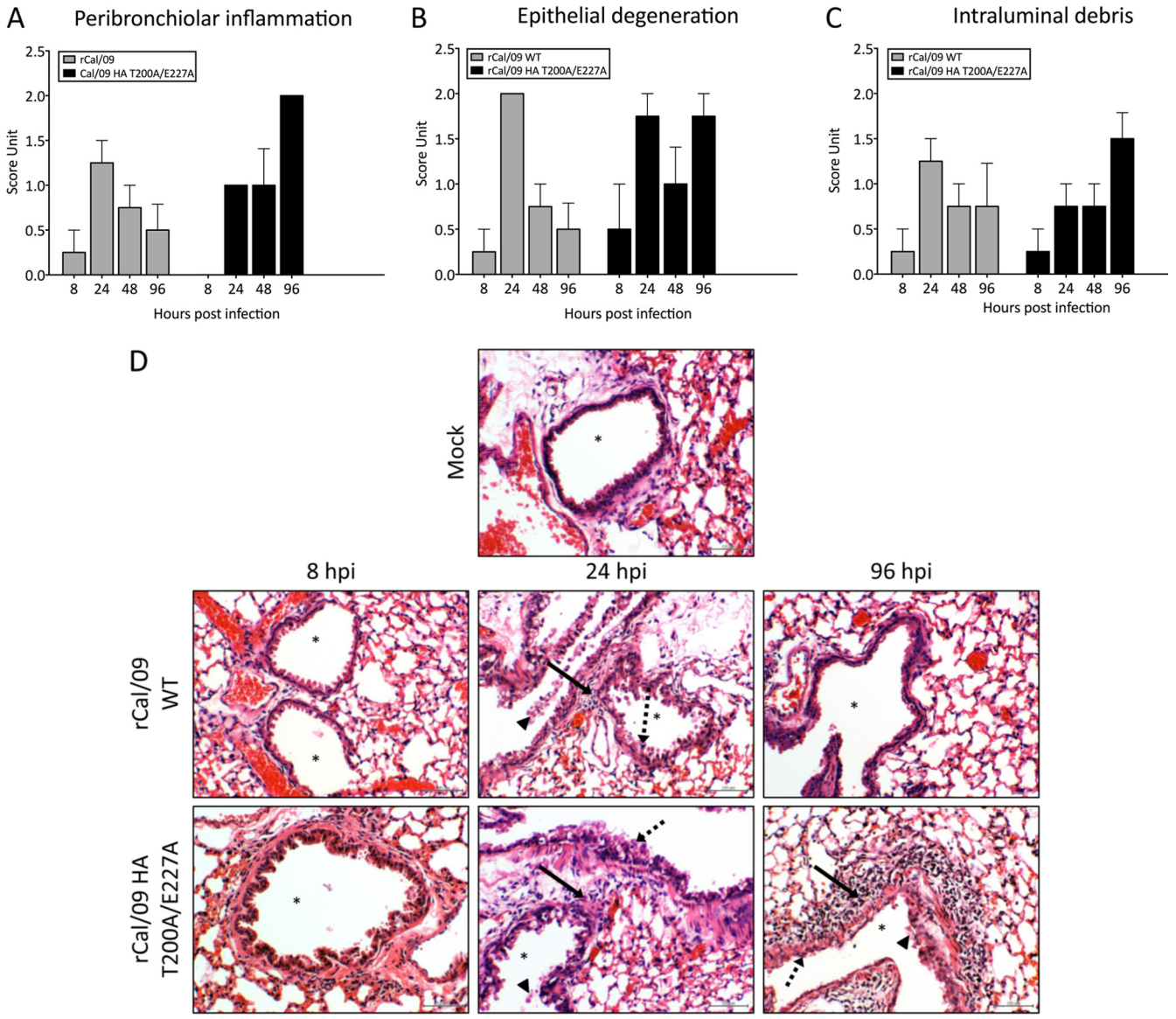

FIG 5 Histopathological analysis of infected mice ($n = 4$) revealed increased bronchiolar lesions associated with rCal/09 HA T200A/E227A virus infection. Peribronchiolar inflammation (A), epithelial degeneration (B), and intraluminal debris (C) were scored as follows: 0, no epithelial degeneration or necrosis, no inflammation; 1, mild, focal, or scattered necrotic cells, cell vacuolation, and scattered inflammatory cells; 2, moderate multifocal cell necrosis, cell vacuolation, and a thin layer of inflammatory cells. (D) Bronchial and bronchiolar areas of hematoxylin-and-eosin-stained mouse lungs inoculated with phosphate-buffered saline (Mock) as a control or infected with the rCal/09 WT and rCal/09 HA T200A/E227A viruses. At 24 h postinfection, bronchioles (*) display different degrees of peribronchiolar inflammation (arrow), epithelial degeneration and necrosis (dotted arrow), and intraluminal debris (arrowhead). At 96 h postinfection, tissue recovery is evident and lesions are receding in rCal/09 WT virus-infected mice. On the contrary, lesions in rCal/09 HA T200A/E227A virus-infected mice do not decrease in time, showing no signs of recovery of the epithelium.

symptoms (sneezing and ruffled fur) were more evident in ferrets infected with the mutant virus.

Our results suggest that substitutions T200A and E227A of Cal/09 influenza virus HA affect viral replication, most likely because of their involvement in receptor binding affinity (6), thus affecting pathogenicity, virulence, and transmission. It is interesting that these mutations allow the virus to replicate to higher levels in MDCK cells and mice, while they delay transmission in ferrets. The molecular reasons for this remain unknown.

The fact that pigs might work as a reservoir for influenza viruses poses a serious threat to the world population, facilitating reassortments among human, swine, and avian strains. Our work contributes to the understanding of how single substitutions in the HA sequence dramatically affect the pathogenesis and transmission of influenza virus. That amino acids 200A and 227A, found in HAs of swine strains, increase viral replication and pathogenesis in mice but decrease transmission in ferrets suggests that specific mutations associated with increased pathogenicity are also associated with decreased transmission, which could reduce the fitness of the virus. Further analysis is needed to investigate the mechanism responsible for these effects.

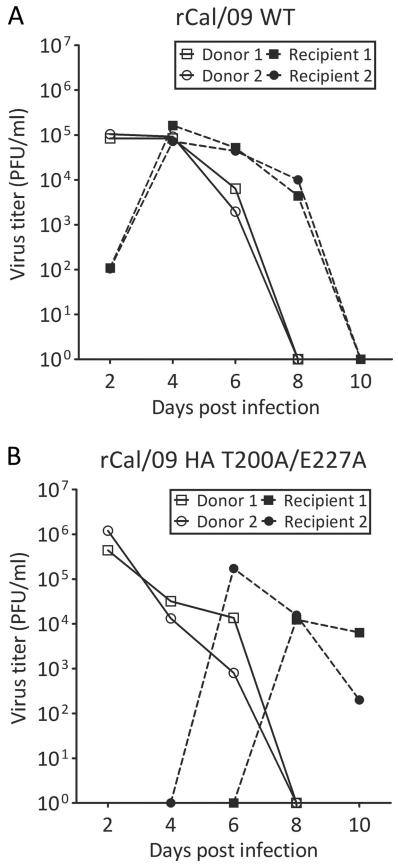

FIG 6 Cal/09 HA T200A/E227A virus transmission is delayed in ferrets. Two groups of ferrets were infected and exposed to rCal/09 WT (A) and rCal/09 HA T200A/E227A (B), respectively. Two animals were inoculated intranasally with the virus, and 1 day later, two naive animals were exposed to aerosol transmission. Virus titers in nasal washes were determined at days 2, 4, 6, 8, and 10 postinfection.

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