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Replication and Pathogenesis of the Pandemic (H1N1) 2009 Influenza Virus in Mammalian Models

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This study aimed to characterize the replication and pathogenic properties of a Korean pandemic (H1N1) 2009 influenza virus isolate in ferrets and mice. Ferrets infected with A/Korea/01/2009 (H1N1) virus showed mild clinical signs. The virus replicated well in lungs and slightly in brains with no replication in any other organs. Severe bronchopneumonia and thickening of alveolar walls were detected in the lungs. Viral antigens were detected in the bronchiolar epithelial cells, in peribronchial glands with severe peribronchitis and in cells present in the alveoli. A/Korea/01/2009 (H1N1) virus-infected mice showed weight loss and pathological lung lesions including perivascular cuffing, interstitial pneumonia and alveolitis. The virus replicated highly in the lungs and slightly in the nasal tissues. Viral antigens were detected in bronchiolar epithelial cells, pneumocytes and interstitial macrophages. However, seasonal H1N1 influenza virus did not replicate in the lungs of ferrets, and viral antigens were not detected. Thus, this Korean pandemic (H1N1) 2009 isolate infected the lungs of ferrets and mice successfully and caused more pathological lesions than did the seasonal influenza virus.

***Keywords*:** pandemic (H1N1) 2009 virus, influenza, ferrets, mice, pathogenesis, immunohistochemistry

In April 2009, a mixed-origin H1N1 influenza virus was recognized as a causative agent of influenza-like illnesses (ILI) in humans. The virus caused outbreaks and fatalities in human populations worldwide and the World Health Organization (WHO) declared an influenza pandemic on June 11, 2009. As of March 26, 2010, this virus had spread to more than 213 countries around the world and more than 16,931 deaths had been reported (http://www.who.int/csr/don/2010_03_26/en/index.html).

The most common symptoms of the pandemic (H1N1) 2009 influenza virus (pandemic 2009 virus) infection in humans are fever, coughing and sore throat, similar to those of seasonal influenza viral infections. However, gastrointestinal distress and vomiting or diarrhea were also reported frequently, which are not typical characteristics of seasonal influenza infections (Dawood *et al*., 2009; Riquelme *et al*., 2009). The pandemic 2009 virus caused uncomplicated febrile respiratory infections ranging from self-limited forms to severe and fatal illness. The viruses are antigenically similar to North American swine H1N1 viruses but distinct from seasonal human H1N1 viruses (Garten *et al*., 2009). Serological studies showed that recent (2005-2009) seasonal influenza vaccines were not likely to elicit a protective antibody response to the pandemic 2009 virus (Centers for Disease Control and Prevention, 2009; Hancock *et al*., 2009). Sequence analyses showed that these viruses had no markers associated with high pathogenicity in avian and/or mammalian species (Itoh *et al*., 2009).

Mammalian models have contributed to the study of pathogenesis and transmission of novel influenza viruses. The ferret (*Mustela putorius furo*) is widely used small animal model for studying the pathogenicity of various kinds of influenza virus. Ferrets are susceptible to natural infections with influenza viruses and develop acute respiratory disease and pathological lung lesions similar to those seen in humans (Maher and DeStefano, 2004; Barnard, 2009). The pathogenicities of avian influenza and novel influenza viruses with pandemic potential were assessed using this model (Zitzow *et al.*, 2002; Govorkova *et al.*, 2005). Mice are not infected by influenza viruses naturally, but are commonly used for the evaluation of pathogenesis and immunity to influenza viruses isolated from humans (Lu *et al.*, 1999; Joseph *et al.*, 2007). The purpose of this study was to characterize the replication and pathogenic properties of the pandemic (H1N1) 2009 virus isolated in Korea, using ferrets and mice.

Materials and Methods

Viruses

A 52-year-old Korean woman who had just returned from Mexico developed upper respiratory symptoms and turned out to be infected by the pandemic 2009 virus on May 2, 2009. A/Korea/01/2009 (A/KR/01) virus was isolated from the throat swabs of the patient and propagated in 10-day-old embryonated chicken eggs. This had a titer

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of $10^{7.4}$ plaque forming units (PFU)/ml. All experiments with the A/KR/01 virus were performed at a biosafety level 3 plus (BSL3+) facilities at the Korea Centers for Disease Control and Prevention (KCDC). The complete genome sequences of A/KR/01 virus with high similarity to A/California/04/2009 have been submitted to GenBank under accession numbers GQ131023-GQ101326, GQ132185, and GQ160811-GQ160813. A seasonal H1N1 virus, A/Solomon Islands/3/2006 (A/Sol/3), was used to compare pathogenesis in ferrets.

Ferret experiments

To evaluate the viral pathogenicity, we used 6∼8-month-old male ferrets (Marshall BioResources, USA), which were serologically negative by hemagglutination inhibition (HI) assays for currently circulating seasonal (A/Sol/3) and pandemic (A/KR/01) influenza viruses. Eight animals were allocated randomly for testing for each virus and baseline body temperature and body weights were measured before inoculation. Temperatures were measured using a subcutaneous implantable temperature transponder (Bio Medic Data Systems, Inc., USA). Ferrets were anesthetized with a cocktail of 25 mg ketamine, 2 mg xylazine and 0.05 mg atropine per kg of body weight, respectively. Six ferrets were inoculated intranasally with 10^7 PFU of A/KR/01 or A/Sol/3 virus and two control animals were mock-inoculated with 1 ml of phosphate-buffered saline (PBS). Three infected ferrets and one mock-infected ferret were euthanized at 3 days post inoculation (dpi) and gross lesions were evaluated. Tissue specimens were collected for viral titration and were immediately frozen on dry ice and stored at -80°C until processing. Virus quantities in the lungs, nasal turbinates, brain, spleen, kidneys, liver, heart, intestine, and blood were measured by plaque assays in Madin-Darby Canine Kidney (MDCK) cells. The remaining four animals were monitored with body weights and body temperature being recorded daily and observed for clinical signs for 14 days. A scoring system of 0 to 3 was used to assess the lethargy of inoculated ferrets and a relative inactivity index (RII) was calculated as described (Reuman *et al*., 1989; Zitzow *et al*., 2002). Nasal washes were collected every other day for 9 days after inoculation and the virus quantity was determined in MDCK cells.

Mouse experiments

Female 6∼7-week-old BALB/c mice (Orient Bio, Korea) were anesthetized with 2,2,2-tribromoethanol in *tert*-amyl alcohol (Avertin; Sigma-Aldrich, USA) and inoculated intranasally with 50 μl of A/KR/01 virus. The fifty percent mouse infectious dose (MID_{50}) and fifty percent mouse lethal dose (MLD_{50}) titers were determined as described (Lu *et al.*, 1999). In brief, eight mice per group were inoculated with serial 10-fold dilutions (from 10^6 to 10^0 PFU) of virus. After 3 days, three mice from each group were euthanized and homogenized lungs were titrated in MDCK cells using a standard plaque assay to determine the MID_{50} calculated by the method of Reed and Muench (Reed and Muench, 1938). The remaining five mice were monitored daily for 14 days for morbidity, as measured by weight loss, and mortality to determine the MLD_{50}. To determine the replication and systemic spread of the virus, six mice were inoculated with 10^5 PFU of virus and three were euthanized on 3 and 6 dpi. At necropsy, whole spleen, heart, kidney, liver, thymus, nose and brain tissues were collected aseptically, immediately frozen on dry ice and stored at -80°C until used. Tissue samples were thawed, homogenized in 1 ml of cold sterile PBS and clarified by centrifugation (2,500×g) at 4°C. Viral titers in homogenates were determined in MDCK cells. All experimental animal work was performed in the BSL3+ animal facilities at KCDC, following approval of the animal safety protocols by the Institutional Animal Care and Use Committee.

Histopathology and immunohistochemistry

For the histopathologic analysis, animals were sacrificed humanely at a designated time (3 dpi for ferrets and 3 and 6 dpi for mice) and tissues (lung, heart, spleen, kidneys, liver, intestine, and brain) were collected and fixed in 10% neutral-buffered formaldehyde, processed routinely and embedded in paraffin wax. They were sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) for light microscopy. For the evaluation of influenza viral antigens, tissues were deparaffinized with xylene and rehydrated through graded alcohols. Endogenous alkaline phosphatase was quenched with 20% glacial acetic acid solution for 10 min at room temperature. All slides were treated with proteinase K (100 μg/ml; Gibco BRL, USA) for 20 min at 37°C and then incubated with normal goat serum for 15 min at room temperature to saturate non-specific protein binding sites. A monoclonal antibody (Novus Biologicals, USA) against the nucleoprotein of influenza A virus was applied and the slides were incubated overnight at 4°C in a humid chamber. After three washes with 0.1% Tween 20 in PBS (PBST), sections were flooded and incubated for 45 min at 37°C with biotinylated rabbit anti-mouse IgG (Dako, Denmark) diluted 1 in 250 in PBST. The slides were then washed with PBST before being flooded and incubated for 30 min at 37°C with a streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals, Germany) diluted 1 in 250 in PBST. They were then equilibrated with Tris buffer (pH 8.2) for 5 min at room temperature. The final reaction was produced by immersing the sections in a solution of red substrate (Boehringer Mannheim, USA) for 10 min at room temperature. The sections were lightly counterstained with Mayer's hematoxylin.

HI assays

Post-exposure sera were collected at 21 dpi from ferrets and at 14 dpi from mice and H1-specific antibodies were evaluated by HI assays using 0.5% turkey red blood cells (RBC). Serum samples were treated with a receptor-destroying enzyme (RDE) from *Vibrio cholerae* (DENKA SEIKEN Co., LTD, Japan) by diluting one part serum with three parts of enzyme and were incubated overnight at 37°C. After heating at 56°C for 30 min they were diluted 1:10 by adding six volumes of PBS. HI assay was performed in 96-well polystyrene plates by twofold serial dilutions of sera in PBS followed by the addition of 4 HA units of inactivated A/KR/01 virus to each well. After 30 min incubation at room temperature, 0.5% turkey RBCs were added to each well and incubated for 30 min. HI titers were determined by the presence of tear-shaped RBC pellets that formed in positive assays after tilting the plates.

Results

Pathogenicity in ferrets

Ferrets inoculated with the A/KR/01 virus were lethargic (RII =1.8) from 4 dpi to 6 dpi but regained some level of activity thereafter. Sneezing was observed frequently from 3 dpi and continued to 6 dpi from three ferrets, and a nasal discharge was observed among two of three ferrets from 3 to 5 dpi. All ferrets lost weight slightly; the mean maximum weight loss was 5.8% and body temperature rose by 1.2°C at 1 dpi (Figs. 1A and B). By contrast, the A/Sol/3 virus inoculated ferrets showed less activity loss (RII=1.0) and sneezing and a nasal discharge were observed from only one ferret. The mean maximum percent weight loss was 5.1% and body temperature

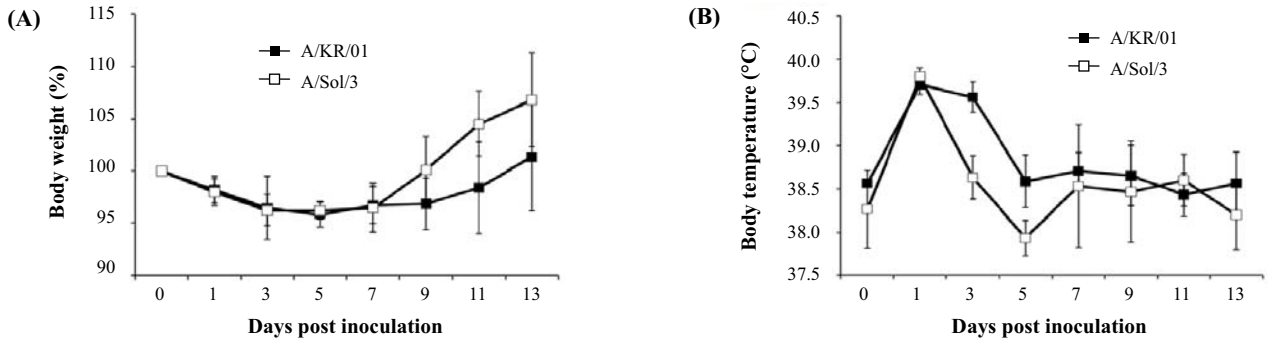

Fig. 1. Body weight and body temperature changes in ferrets inoculated with seasonal and pandemic (H1N1) 2009 influenza virus. Three ferrets in each group were inoculated intranasally with 10^7 PFU of virus and body weights (A) and body temperatures (B) were monitored.

rose by 1.5°C, which was similar to those caused by A/KR/01 (Figs. 1A and B). The pandemic 2009 virus replicated highly in the upper and lower respiratory tract of ferrets; however, the seasonal H1N1 virus replicated only in the upper respiratory tract (Table 1). In particular, virus was detected in brains at a low titer from two (olfactory bulb) and one (anterior brain) of three pandemic 2009-infected ferrets and one (olfactory bulb) of three seasonal H1N1 virus-infected animals (Table 1). No virus was recovered from the heart, liver, kidney, spleen, thymus, intestine or blood from pandemic 2009 or seasonal H1N1 virus infected ferrets by plaque assays. In the nasal washes of ferrets, both viruses showed similar viral shedding patterns; however, A/KR/01 virus-inoculated ferrets shed for longer periods with much higher titers of the virus than did the A/Sol/3-inoculated ferrets (Table 1). Serum neutralizing antibodies against both A/KR/01 and A/Sol/3 viruses were elevated to 1:640 to 1:1,280 at 21 dpi by HI assays (Table 2).

Ferret lung pathology

At necropsy, focal areas of redness and consolidation of the lungs were evident in A/KR/01 virus-infected ferrets but other extrapulmonary gross lesions were not seen. Histopathologically, ferret lung tissues showed severe bronchopneumonia with inflammatory cell infiltrates composed predominantly of neutrophils, and thickening of the alveolar walls (Fig. 2A). We found multifocal microglial nodules in brain tissues (Fig. 2B). Influenza A viral antigen expression was examined, and bronchial, bronchiolar epithelial cells and desquamated cells in the bronchiolar lumen were found to be infected by the pandemic (H1N1) 2009 influenza virus (Fig. 2C). Pneumocytes and cells in the thickened alveolar wall were stained with an influenza A-specific nucleoprotein (NP) antibody (Figs. 2D and E) and extensive viral antigen immunostaining was detected in the peribronchial glands, with severe peribronchitis (Fig. 2F). In contrast, A/Sol/3 virus-infected ferrets showed mild bronchopneumonia and interstitial pneumonia; however, no microscopic lesions were found in brains. No viral antigen was detected in lungs by immunohistochemistry (Fig. 2G). Influenza A viral antigen was not detected in any other extrapulmonary tissues in ferrets inoculated with the A/KR/01 or A/Sol/3 viruses by immunohistochemistry.

Pathogenicity in mice

The A/KR/01 virus-infected mice showed weight loss (Fig. 3) and the MID_{50} on 3 dpi was $10^{1.5}$ PFU. However, all mice survived during the period of the experiment at the highest dose tested ($MLD_{50}>10^6$ PFU). High titers of pandemic 2009 virus were detected in the lungs of inoculated mice on 3 and 6 dpi at $10^{6.4}$ PFU/ml and $10^{5.7}$ PFU/ml, respectively. At 3 dpi, two of three mice had low titers of virus in the nose ($10^{2.8}$ PFU/ml) and one of three mice had a high titer in the heart ($10^{5.9}$ PFU/ml). No virus was detected in the liver, kidney, spleen or brain. To evaluate the viral infectivity to the BALB/c mice, serial 10-fold dilutions (10^0-10^6 PFU) of virus were inoculated and lung viral titers were determined at 3 dpi. The virus replicated in the lungs of mice inoculated with 10^2 PFU and mice inoculated with 10^3 or 10^4 PFU had much higher titers than any other groups (Table 3). Mice inoculated with 10^4 to 10^6 PFU of virus lost body weight, whereas those inoculated with 10^2 or 10^3 PFU gained weight (Fig. 3). Serum antibody titers of serial 10-fold dilutions of virus-inoculated mice were elevated from 1:10 to 1:320 at 14 dpi by HI assays

Table 1. Viral titers in organs and nasal washes of ferrets inoculated with the pandemic (H1N1) 2009 or seasonal H1N1 virus

Virus	Viral titer (mean $\log_{10}$ PFU±SD/g)[a]					Viral titer (mean $\log_{10}$ PFU±SD/ml)[b]			
	Respiratory tract		Brain			Nasal wash			
	Nasal turbinates	Lung	Olfactory bulb	Anterior brain	Posterior brain	Day 1	Day 3	Day 5	Day 7
A/KR/01	7.5±0.2	6.7±0.4	2.0/3.6	3.9	–[c]	8.4±0.1	5.9±0.3	3.1±0.4	–
A/Sol/3	4.7±0.3	–	3.2	–	–	6.3±0.7	4.1±0.7	–	–

[a] Three ferrets were euthanized on the third day post inoculation (3 dpi) for viral titration. When viruses were recovered from all three ferrets, mean titers are presented. When viruses were not recovered from all animals, individual titers are shown.
[b] Nasal washes from three ferrets were collected every other day for viral titration.
[c] Virus not detected (detection limit: 1.5 $\log_{10}$ PFU/g of tissue).

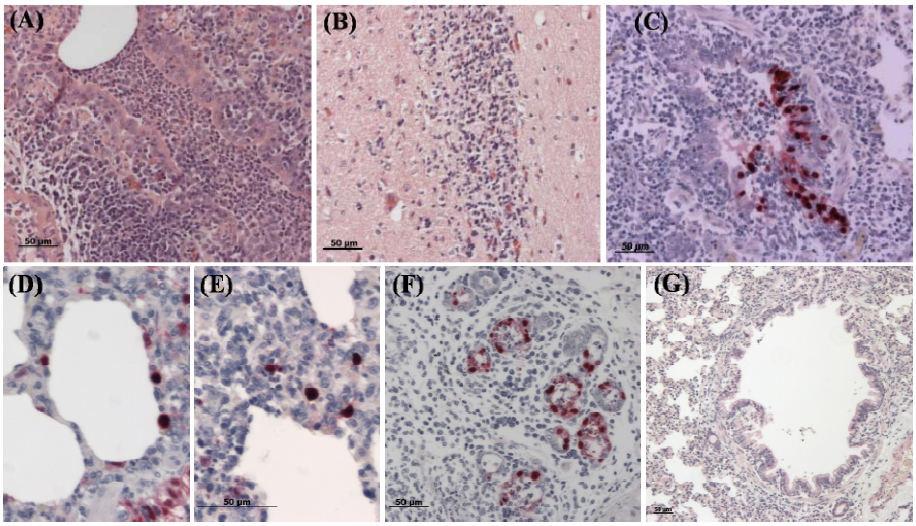

Fig. 2. Representative histopathology and immunohistochemical results in tissues from ferrets inoculated with the A/KR/01 (A-F) or A/Sol/3 (G) virus. Tissues were collected on 3 dpi and were processed for hematoxylin and eosin (H&E) and immunohistochemical staining. (A) H&E staining of the lung showing extensive bronchiolar inflammation, necrosis of the bronchiolar epithelium and suppurative exudates in the bronchiolar lumen. (B) H&E staining of the brain showing glial nodules. (C-F) Immunohistochemical staining of the A/KR/01 virus-infected lung. (C) Extensive viral antigen was detected in the bronchial epithelial cells. (D) and (E) Sparse viral antigen was detected in pneumocytes and interstitial macrophages in thickened alveolar walls. (F) Extensive viral antigen was detected in the peribronchial glands. (G) Immunohistochemical staining of A/Sol/3 virus-infected lung. Viral antigen was not detected.

(Table 3).

Mouse lung pathology

A/KR/01 virus-infected mouse lung tissues showed multifocal mild to moderate necrotizing bronchiolitis and interstitial pneumonia. Prominent alveolar thickening with extensive inflammatory infiltrates was detected at 3 dpi (Fig. 4B) and perivascular cuffing with accumulation of lymphocytes around the blood vessels was found (Fig. 4C). The microscopic lung lesions of mice at 6 dpi were more severe than those at 3 dpi (Fig. 4D). Positive staining by immunohistochemistry in tissues of mice was confined to the respiratory tract. Viral antigen was detected extensively in bronchiolar epithelial cells and in desquamated cells in the bronchiolar lumen. Viral antigen was detected in pneumocytes and cells in thickened interstitial septa (Figs. 4E and F). Much more viral antigen was detected in lungs of A/KR/01 virus-infected mice at 3 dpi than at 6 dpi. Influenza A virus-specific antigen was not detected in any other extrapulmonary tissues.

Table 2. Serum antibody titers of ferrets inoculated with A/KR/01 or A/Sol/3 viruses

Virus	HI titer[a]		
	Before inoculation	14 dpi	21 dpi
A/KR/01	<10/<10/<10	640/640/640	1280/640/1280
A/Sol/3	<10/<10/<10	320/640/640	640/1280/640

[a] Sera were taken from three ferrets at 14 and 21 dpi. Antibody titers against A/KR/01 or A/Sol/3 viruses were determined by hemagglutination inhibition (HI) assays using 0.5% turkey erythrocytes. Titers for each ferret are shown.

Discussion

The pathogenesis and transmission of several strains of the pandemic (H1N1) 2009 virus have been studied in animal models including ferrets, mice, macaques and pigs (Itoh *et al*., 2009; Lange *et al*., 2009; Maines *et al*., 2009; Munster *et al*., 2009; Brookes *et al*., 2010). The pathogenic properties in mammalian models were similar, but they have shown some differences depending on the isolates.

In this study, we demonstrated the replication and pathogenicity of Korean pandemic 2009 virus, A/KR/01, in

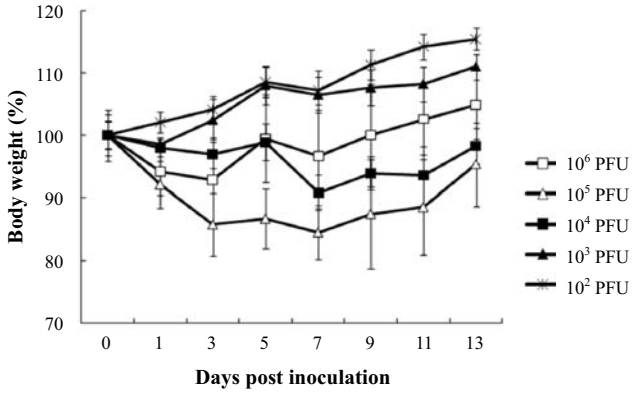

Fig. 3. Body weight changes in A/KR/01 virus-infected mice. Five mice per group were inoculated intranasally with serial dilutions of A/KR/01 virus (10^2-10^6 PFU), and body weights were monitored every other day. The values are mean percentage changes and error bars represent standard deviations of the mean.

Table 3. Viral titers in lungs and serum antibody titers of A/KR/01 virus-inoculated mice

Amount	Viral titers (mean $\log_{10}$ PFU±SD/g)[a]	HI titer[b]
10^6 PFU	5.9±0.2	320
10^5 PFU	6.4±0.4	320
10^4 PFU	7.5±0.1	320
10^3 PFU	7.4±0.4	160
10^2 PFU	5.8±0.1	80
10^1 PFU	–[c]	10
10^0 PFU	–	<10

[a] BALB/c mice were inoculated intranasally with 10^0-10^6 plaque forming units (PFU) of virus. Three mice from each group were euthanized on 3 dpi for viral titration.
[b] Sera were taken from five mice at 14 dpi and were pooled. Antibody titers against A/KR/01 virus were determined by hemagglutination inhibition (HI) assays using 0.5% turkey erythrocytes.
[c] Virus not detected (detection limit:1.7 $\log_{10}$ PFU/ml).

ferrets and mice. Our results indicated that this virus replicated more efficiently in the upper (nasal turbinates) and lower (lungs) respiratory tract of ferrets than did the A/Sol/3 virus, and these findings are in agreement with previous observations (Itoh *et al*., 2009; Maines *et al*., 2009; Munster *et al*., 2009). The seasonal influenza virus did not replicate in lungs of inoculated ferrets and no antigen was found by immunohistochemistry; these results are in accordance with those of other studies (Itoh *et al*., 2009; Munster *et al*., 2009). Although both viruses presented with similar viral shedding patterns, much higher titers of virus were found and less physical activity was demonstrated in the A/KR/01 virus-infected ferrets. In terms of body weight change, A/Sol/3 virus-inoculated ferrets recovered their weight much faster than did the A/KR/01 virus-inoculated ferrets after 7 dpi (Fig. 1A). Therefore, the A/KR/01 virus seemed to be more pathogenic than the seasonal human H1N1 virus, A/Sol/3.

We found a different pathogenic property of the A/KR/01 virus compared with other pandemic 2009 viruses in ferrets. Histopathologically, multifocal glial nodules were seen in brain tissue and this was supported by the replication of virus in the olfactory bulb and anterior part of the brain. Although viral protein was not detected in brains by immunohistochemistry, these data suggest that the pandemic 2009 virus reached the brains of infected ferrets and had the potential for causing more systemic lesions including encephalopathy than did the seasonal H1N1 virus.

Human strains of influenza A virus which cause seasonal epidemics do not typically replicate efficiently in the mouse model; however, they can replicate efficiently and acquire virulence after adaptation by serial lung-to-lung passages (Hartley *et al*., 1997). The A/KR/01 virus caused weight loss and replicated highly in the lungs of mice without prior host adaptation, and the MID_{50} titer was markedly low. These results indicate the high infectivity of this virus in a murine model. A high titer of infectious virus was demonstrated in the heart of one of three mice tested. Because viral antigen was not detected in heart tissues, we cannot exclude the possibility of contamination with viruses originating from the lungs, which were severely affected and had high viral titers from the infection. Although the virus replicated efficiently in the lungs of mice, it did not always produce clinical signs. Our data show that mice inoculated with 10^2 and 10^3 PFU had high viral titers in the lungs and elevated antibody titers in the blood, but they did not lose weight (Fig. 3). After the waves of the 2009 pandemic, a high seroprevalence of antibodies against the pandemic 2009 virus in the Iranian population suggests

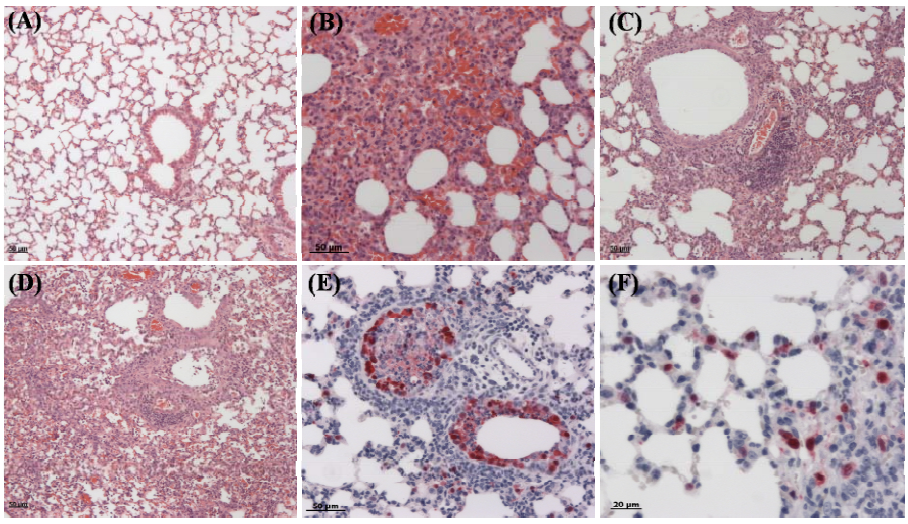

Fig. 4. Representative histopathology and immunohistochemical results in lungs from mice inoculated with the A/KR/01 virus. Tissues were removed on 3 and 6 dpi and were processed for H&E (A-D) and immunohistochemical staining (E-F). (A) Mock-infected mouse lung showing normal histology. (B-F) A/KR/01 virus-infected mice. (B) Prominent alveolar thickening was seen with extensive inflammatory infiltrates at 3 dpi. (C) Perivascular cuffing and mild to moderate alveolitis was observed. (D) More severe lung lesions were observed at 6 dpi. (E) and (F) Immunohistochemical staining of A/KR/01 virus-infected mouse lung at 3 dpi. (E) Viral antigen-positive cells were detected in the bronchiolar epithelial cells and desquamated cells in the bronchiolar lumen. (F) Viral antigen was detected in pneumocytes and interstitial cells.

either a high level of pre-existing immunity or a high rate of asymptomatic infection (Moghadami *et al*., 2010). Our data support the possibility of an asymptomatic human infection by the pandemic 2009 virus.

To better understand the pathogenesis of the pandemic 2009 virus, it is important to identify the replication sites in tissues. The major replication sites of the A/KR/01 virus in ferrets were bronchial and bronchiolar epithelial cells, pneumocytes and alveolar macrophages. We also found viral antigens in peribronchial glands associated with inflammation and in desquamated cells in the bronchiolar lumen. Likewise, the A/KR/01 virus replicated mainly in bronchial and bronchiolar epithelial cells and in pneumocytes in mice. Alveolar macrophages also showed positive signals. Microscopic lung lesions in the mice at 3 dpi were less severe than those at 6 dpi; however, much more positive cells were found in mouse lungs. These suggest that the active replication of the pandemic 2009 virus in its acute phase occur within 3 dpi and that severe lung damage thereafter is caused by other factors such as cytokines and chemokines. Although we could not perform transmission experiments in ferrets, recent studies show the efficient transmission of the virus, which explains the rapid transmission of this pandemic 2009 virus between humans by the status of worldwide outbreaks. For the effective control and prevention of new variants of the pandemic (H1N1) 2009 virus, we need to characterize the replication, pathogenicity, and transmission of these viruses in animal models.

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