MAJOR ARTICLE

Severity of Pneumonia Due to New H1N1 Influenza Virus in Ferrets Is Intermediate between That Due to Seasonal H1N1 Virus and Highly Pathogenic Avian Influenza H5N1 Virus

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(See the editorial commentary by Whitley, on pages 976–977.)

Background. The newly emerged influenza A(H1N1) virus (new H1N1 virus) is causing the first influenza pandemic of this century. Three influenza pandemics of the previous century caused variable mortality, which largely depended on the development of severe pneumonia. However, the ability of the new H1N1 virus to cause pneumonia is poorly understood.

Methods. The new H1N1 virus was inoculated intratracheally into ferrets. Its ability to cause pneumonia was compared with that of seasonal influenza H1N1 virus and highly pathogenic avian influenza (HPAI) H5N1 virus by using clinical, virological, and pathological analyses.

Results. Our results showed that the new H1N1 virus causes pneumonia in ferrets intermediate in severity between that caused by seasonal H1N1 virus and by HPAI H5N1 virus. The new H1N1 virus replicated well throughout the lower respiratory tract and more extensively than did both seasonal H1N1 virus (which replicated mainly in the bronchi) and HPAI H5N1 virus (which replicated mainly in the alveoli). High loads of new H1N1 virus in lung tissue were associated with diffuse alveolar damage and mortality.

Conclusions. The new H1N1 virus may be intrinsically more pathogenic for humans than is seasonal H1N1 virus.

A new influenza A virus of the subtype H1N1 (new H1N1 virus) that first emerged in Mexico at the beginning of 2009 is the first pandemic human influenza virus of the 21st century. Studies have concluded that this virus probably originated from a domestic swine reservoir on the basis of its genetic signature [1, 2]. On 11 June 2009, the World Health Organization declared phase 6 of the global pandemic alert level [3]. As of 25 August 2009, more than 182,166 cases, including 1799 deaths, in 177 countries have been officially reported [4].

The main concerns about a new influenza pandemic are the disease burden and associated mortality it may cause. These are largely due to pneumonia resulting from extension of the viral infection to the lower respiratory tract. The initial lung damage may be complicated by concomitant or subsequent bacterial pneumonia, which has been shown to be a major cause of mortality during the Spanish influenza epidemic in 1918, before the use of antibiotics [5]. In the current outbreak of new H1N1 influenza, bacterial coinfection was registered in only 20% of the cases investigated [6]. In contrast to the damage caused by the virus infection to the tracheobronchial epithelium in uncomplicated influenza, damage to the alveolar epithelium has severe consequences for the gas exchange function of the respiratory tract. It allows fluid from the alveolar capillaries to flood the alveolar lumina, causing severe—and in some cases fatal—respiratory dysfunction [7].

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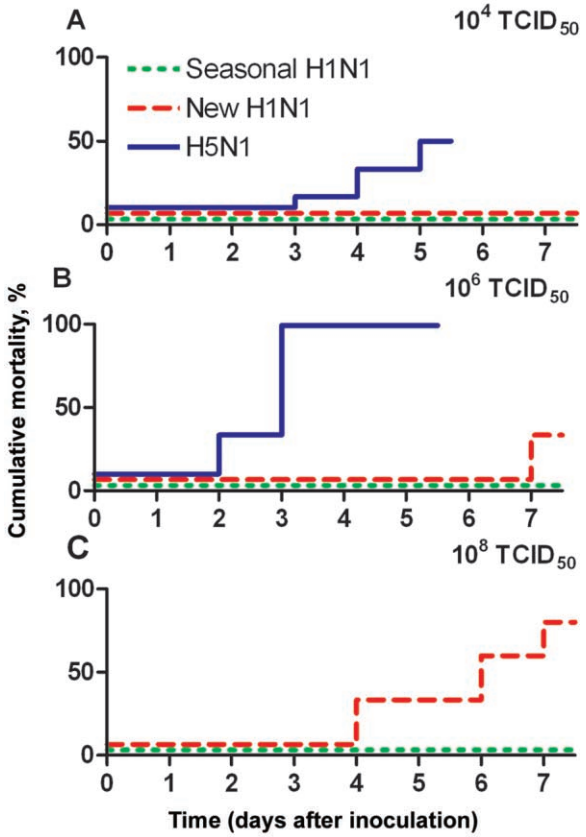

Figure 1. Cumulative mortality rates of ferrets inoculated with different influenza viruses. Ferrets were intratracheally inoculated with seasonal H1N1 ($n = 3$), new H1N1 ($n = 6$), or highly pathogenic avian influenza (HPAI) H5N1 ($n = 6$) influenza viruses at a dose of 10^4 (*A*), 10^6 (*B*), or 10^8 (*C*) median tissue culture infective dose ($TCID_{50}$). Cumulative mortality for new H1N1 virus was intermediate between that for seasonal H1N1 virus and that for HPAI H5N1 virus.

On the basis of the observed relatively low case fatality rate in humans, it is assumed that the virulence of the new H1N1 virus is low. However, it is acknowledged that these figures are uncertain because of too limited data [8]. Therefore, the potential of the new H1N1 virus to cause lower respiratory tract disease in humans is still poorly understood. In a previous publication, we used intranasal inoculation of influenza virus into ferrets mainly to model the transmission of the new H1N1 virus in humans [9]. Here, we use intratracheal inoculation of influenza virus into ferrets as a model of influenza pneumonia in humans. This intratracheal route of inoculation is an appropriate model of infection in humans, which is commonly thought to occur through inhalation of infectious droplets or airborne droplet nuclei [10]. We have used this route of inoculation successfully in previous studies involving both ferrets [11] and macaques [12] to model viral pneumonia. Our results showed that the severity of pneumonia caused by the new H1N1 virus was intermediate between that caused by seasonal H1N1 virus and by highly pathogenic avian influenza (HPAI) A(H5N1) virus.

METHODS

Virus preparation. Three viruses were used: the new H1N1 virus (A/Netherlands/602/2009), isolated from a specimen from a human patient who had recently visited Mexico; seasonal H1N1 virus (A/Netherlands/26/2007), isolated from a patient during the 2006–2007 influenza season; and HPAI H5N1 virus (influenza A/Indonesia/5/2005), as described elsewhere [9].

Study design. For the survival study, groups of 3 ferrets (for seasonal H1N1 virus) or 6 ferrets (for new H1N1 and HPAI H5N1 viruses) with a temperature logger in the peritoneal cavity were inoculated intratracheally with each of these 3 viruses at each of the following doses in a 3-mL volume: 10^4, 10^6, and 10^8 median tissue culture infective dose ($TCID_{50}$) of

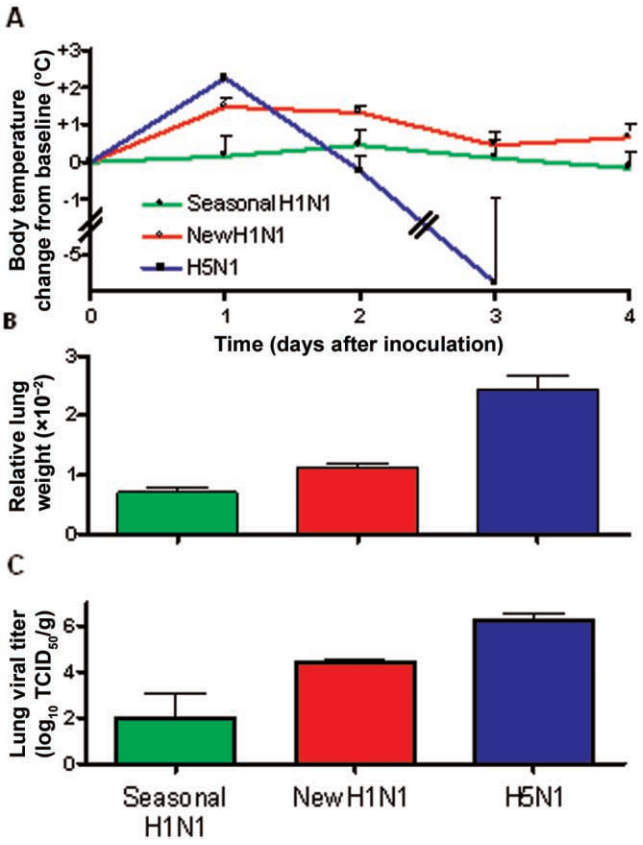

Figure 2. Body temperatures, relative lung weights and lung viral titers of ferrets inoculated with different influenza viruses. The ferrets were intratracheally inoculated with seasonal H1N1 ($n = 3$), new H1N1 ($n = 6$) or highly pathogenic avian influenza (HPAI) H5N1 ($n = 6$) influenza viruses at a dose of 10^6 median tissue culture infective dose ($TCID_{50}$). The increase in body temperature (*A*), the relative lung weight (*B*), and the lung viral titer (*C*) of the new H1N1 virus group were intermediate between those of the seasonal H1N1 virus group and that of the HPAI H5N1 virus group.

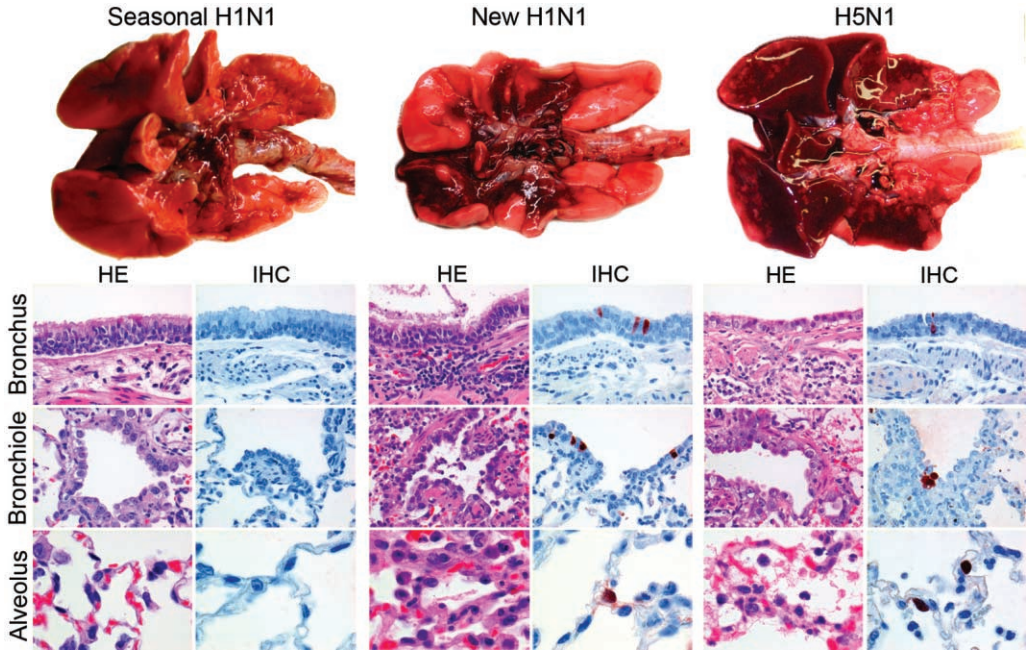

Figure 3. Macroscopy, histopathology, and immunohistochemistry in the lungs of ferrets inoculated with different influenza viruses. The severity of macroscopic lung lesions (*top row*) in the new H1N1 virus group were intermediate between those in the seasonal H1N1 and the highly pathogenic avian influenza (HPAI) H5N1 virus groups. The new H1N1 virus group showed moderate influenza virus expression (IHC) in bronchi, bronchioles, and alveoli, associated with histological lesions (HE) characterized by inflammatory cell infiltrates and epithelial necrosis. In contrast, the seasonal H1N1 virus group showed minimal influenza virus expression and histological lesions. In the HPAI H5N1 virus group, there was more abundant influenza virus antigen expression in the alveoli associated with more severe histological lesions, but less abundant influenza virus antigen expression in the bronchi and bronchioles, associated with milder histological lesions. Original magnification, bronchus and bronchiole, ×400, and alveolus, ×1000. HE, hematoxylin-eosin; IHC, immunohistochemistry with 3-amino-9-ethylcarbazole substrate and hematoxylin counterstain.

virus. After inoculation, they were monitored daily for clinical signs until maximally 7 days after inoculation [11]. For the pneumonia study, groups of 3 ferrets (for seasonal H1N1 virus) or 6 ferrets (for new H1N1 and HPAI H5N1 viruses) were inoculated intratracheally with 10^6 $TCID_{50}$ of virus and euthanized at 4 days after inoculation or earlier when they were moribund before that time point. Necropsies were performed, and samples were obtained from both respiratory and extrarespiratory tissues for virological, pathological, and immunohistochemical analyses.

Ferrets. The experiments were performed under biosafety level 3 conditions at the Netherlands Vaccine Institute under an animal study protocol approved by the Institutional Animal Welfare Committee. Thirteen-month-old purpose-bred ferrets that were seronegative for antibody against circulating influenza viruses and Aleutian disease virus were maintained in standard housing and provided with commercial food pellets and water ad libitum until the start of the experiment. All ferrets were female (body weight, 815–1110 g) except for 1 male each in the new H1N1 virus and seasonal H1N1 virus groups of the pneumonia study (body weight, 1150 and 1290 g, respectively). Two weeks before infection, the animals were anesthetized using a cocktail of ketamine (Nimatek; Eurovet Animal Health BV) and domitor (Orion Pharma), and a temperature logger (data storage tag micro-T ultra small temperature logger; Star-Oddi) was placed in the peritoneal cavity. This device recorded the body temperature of the animals every 15 min.

Pathology. Three animals from each group were euthanized by exsanguination under ketamine anesthesia at 4 or 7 days after inoculation, and necropsies were performed according to a standard protocol. The trachea was clamped off so that the lungs would not deflate upon opening the pleural cavity, which allowed visual estimation of the area of affected lung parenchyma. Samples for histological examination were stored in 10% neutral-buffered formalin (lungs after inflation with formalin), embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin for examination by light microscopy. The following tissues were examined by light microscopy: left lung (cranial and caudal lobe), nasal turbinate, nasal septum, larynx, trachea, bronchus, tracheobronchial lymph node, eyelid, tonsil, heart, liver, spleen, kidney, pancreas, duodenum, jejunum, colon, adrenal gland, and brain. Samples were obtained from the lungs in a standardized way, not guided by changes as seen in the gross pathology.

Semiquantitative assessment of influenza virus–associated inflammation in the lung was performed as reported elsewhere

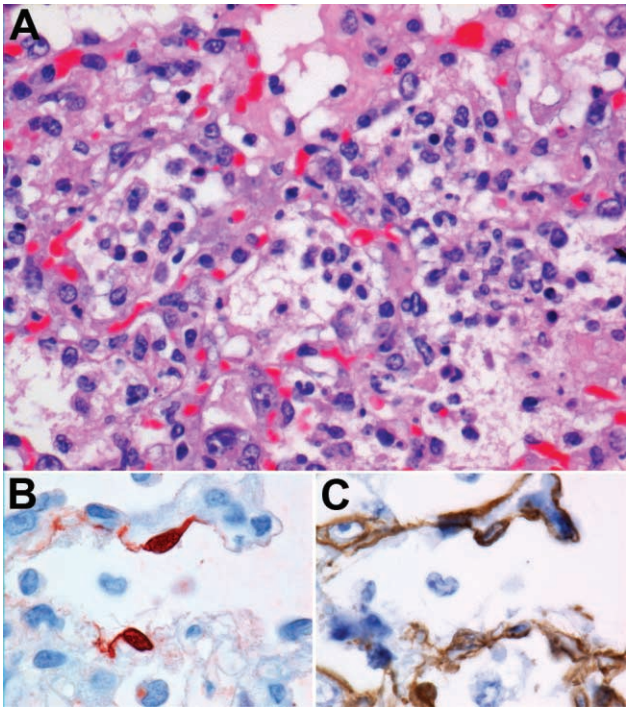

Figure 4. Diffuse alveolar damage in a ferret inoculated with new H1N1 virus. *A*, The alveolar architecture is obliterated by thickening of alveolar septa and flooding of alveolar lumina with alveolar macrophages, neutrophils, and erythrocytes, mixed with fibrin, edema fluid, and cellular debris. Hematoxylin-eosin, original magnification, ×400. *B*, Influenza virus antigen expression in nucleus and cytoplasm of type 1 pneumocytes. Immunohistochemistry with 3-amino-9-ethylcarbazole substrate and hematoxylin counterstain. Original magnification, ×1000. *C*, Keratin expression in cytoplasm of identical cells that expressed influenza virus antigen (shown in panel *B*), confirming epithelial origin of infected cells. Immunohistochemical analysis with diaminobenzidine substrate and hematoxylin counterstain. Original magnification, ×1000 (panel *C*).

[13] for the alveoli. For the degree of inflammation in the bronchi and bronchioles, we used (1) <10% of the bronchial and bronchiolar epithelium inflamed, (2) 10%–50% of the bronchial and bronchiolar epithelium inflamed, and (3) >50% of the bronchial and bronchiolar epithelium inflamed. For the severity of inflammation in the bronchi and bronchioles, we scored (1) peribronchial and peribronchiolar infiltrates, (2) mild necrosis and moderate numbers of inflammatory cells with scant exudate, and (3) marked necrosis and large numbers of inflammatory cells with abundant exudate. Microscopy slides were examined without knowledge of the identity of the animals. The cumulative scores for size and severity of inflammation provided the total score per animal.

Immunohistochemical analysis. For detection of influenza A virus antigen, tissues were stained with a primary antibody against the influenza A nucleoprotein as described elsewhere [14]. Semiquantitative assessment of influenza virus antigen expression in the lungs was performed as reported elsewhere [13] for the alveoli. For the bronchi and bronchioles, the percentage of positively staining bronchial and bronchiolar epithelium was estimated on every slide, and the average of the 4 slides was taken to provide the score per animal. For phenotyping of alveolar epithelial cells we used a destaining-restaining technique described elsewhere [15].

Virology. Virus titers were determined by virus isolation in Madin-Darby canine kidney cells. Upon necropsy, samples of ~0.1 g of the cranial, median, and caudal lobe of the right lung and of the accessory lobe from each animal were collected (total average weight of ~0.4–0.5 g per animal), pooled and homogenized with a FastPrep homogenizer (MP Biomedicals) in 3 mL transport medium. Quadruplicate 10-fold serial dilutions of these samples were cultured on Madin-Darby canine kidney cells [16]. Samples were obtained from the lungs in a standardized way, not guided by changes as seen in the gross pathology.

Statistical analysis. We used 1-way analysis of variance (ANOVA) and Tukey multiple comparisons of means to compare body temperature, relative lung weight, lung virus titer, percentage of affected lung parenchyma, and histological and immunohistochemical scores at different levels of the lower respiratory tract for comparing the scores of pulmonary lesions and viral antigen expression. Differences were considered significant at $P < .05$.

RESULTS

The cumulative mortality rate of ferrets inoculated with the new H1N1 virus increased progressively from 0% at a dose of 10^4 $TCID_{50}$ to 80% at a dose of 10^8 $TCID_{50}$ and was intermediate between the cumulative mortality rate for infection with seasonal H1N1 virus and that for infection with HPAI H5N1 virus (Figure 1). On the basis of these data, the estimated median lethal dose was ~10^4 $TCID_{50}$ for the HPAI H5N1 virus, ~10^7 $TCID_{50}$ for the new H1N1 virus, and >10^8 $TCID_{50}$ for the seasonal H1N1 virus. Because the inoculation dose of 10^6 $TCID_{50}$ allowed the best discrimination in cumulative mortality rate between the 3 viruses, we performed additional comparative clinical, pathological, and virological studies on ferrets inoculated with this dose.

Clinical signs were observed in all ferrets inoculated with 10^6 $TCID_{50}$ of the new H1N1 virus from 1 day after inoculation onward; these signs included lethargy, loss of appetite, dyspnea, and raised body temperature (Figure 2*A*). In contrast, the seasonal H1N1 virus group showed no obvious clinical signs at any time point after inoculation. The body temperature in the new H1N1 virus group was significantly higher than that in the seasonal H1N1 virus group at 1 day ($P = .02$) and 2 days after inoculation ($P = .04$; Tukey test). The HPAI H5N1 virus group showed more severe clinical signs than did the new H1N1 virus group, and all ferrets died or were euthanized because of

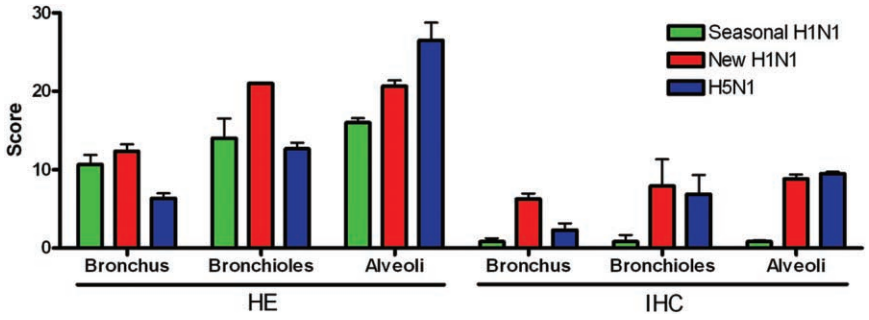

Figure 5. Histological and immunohistochemical scoring in the lungs of ferrets inoculated with different influenza viruses. Histological scoring of samples stained with hematoxylin-eosin (HE) showed that the alveolar lesions in the new H1N1 virus group were intermediate in severity between those of the seasonal H1N1 virus group and the highly pathogenic avian influenza (HPAI) H5N1 virus group, and that the bronchiolar lesions in the new H1N1 virus group were the most severe of all 3 groups. Scoring of the immunohistochemical analysis (IHC) showed that influenza virus antigen expression in the new H1N1 virus group was high in alveoli, bronchioles, and bronchi. In the HPAI H5N1 virus group, the scores were highest for alveoli and lower in bronchioles and bronchi. In the seasonal H1N1 virus group, scores were low at all 3 levels.

their moribund state by 3 days after inoculation. The increase in body temperature of the HPAI H5N1 virus group was comparable to that in the new H1N1 virus group at 1 day after inoculation; at later time points, it decreased due to the progressively moribund state of the ferrets. The mean loss in body weight ± standard deviation was 14% ± 5.3% in the new H1N1 virus group and 8% ± 5.6% in the seasonal H1N1 virus group at 4 days after inoculation. Sneezing was not observed in any of the ferrets.

At necropsy on day 4 after inoculation, the relative lung weights (Figure 2*B*) and the lung virus titers (Figure 2*C*) of the new H1N1 virus group were intermediate between those of the seasonal H1N1 virus and those of the HPAI H5N1 virus group. The relative lung weights among the 3 virus groups were significantly different ($P = .001$; ANOVA, $F = 18.1$), with the relative lung weight of the HPAI H5N1 virus group significantly higher ($P = .006$; Tukey test) than that of the new H1N1 virus group. Lung virus titers among the 3 virus groups were significantly different ($P = .001$; ANOVA, $F = 17.4$), with lung viral titer of the new H1N1 virus group significantly higher ($P = .05$; Tukey test) than that of the seasonal H1N1 virus group.

All ferrets inoculated with the new H1N1 virus had multifocal or coalescing pulmonary lesions, which were dark red, raised, and firmer than normal (Figure 3). The percentage of lung parenchyma affected by this consolidation among the 3 virus groups was significantly different ($P = .0003$; ANOVA, $F = 42.5$). The percentage of affected lung parenchyma in the new H1N1 virus group (range, 20%–70%) was intermediate between that of the seasonal H1N1 virus group (range, 0%–10%; $P = .002$; Tukey test) and that of the HPAI H5N1 virus group (80%–100%; $P = .01$; Tukey test).

On histopathological examination, the alveoli of both the new H1N1 virus group and the HPAI H5N1 virus group showed diffuse alveolar damage (Figures 3 and 4*A*), whereas the alveoli of the seasonal H1N1 virus group did not. This lesion was characterized by flooding of alveolar lumina with alveolar macrophages, neutrophils, and erythrocytes, mixed with fibrin, edema fluid, and cellular debris. The alveolar walls were thickened and had necrosis of the lining epithelium and multifocal type 2 pneumocyte hyperplasia. Qualitatively, the alveolar parenchyma of the new H1N1 virus group had more infiltration by inflammatory cells, whereas that of the HPAI H5N1 virus group had more necrosis and edema.

The bronchiolar epithelium of the new H1N1 virus group had multifocal necrosis with moderate infiltrates of neutrophils and multifocal peribronchiolar infiltration of moderate numbers of macrophages, lymphocytes, and few neutrophils and plasma cells. In the bronchiolar lumen, there were moderate numbers of macrophages, neutrophils, and erythrocytes, mixed with fibrin, edema fluid, and cellular debris. Few bronchi had peribronchial infiltrates with moderate numbers of lymphocytes, macrophages, and few plasma cells and macrophages; in the bronchial lumina were moderate amounts of cellular debris, fibrin, edema and few neutrophils. In the HPAI H5N1 and seasonal H1N1 virus groups, the bronchiolar and bronchial lesions were less severe overall than those in the new H1N1 virus group.

Quantitative histological scoring showed that the alveolar lesions in the new H1N1 virus group were intermediate in severity between those of the seasonal H1N1 virus group and those of the HPAI H5N1 virus group, and that the bronchiolar lesions in the new H1N1 virus group were the most severe of all 3 groups (Figure 5). The histological scores among the 3 virus groups differed significantly for the alveoli ($P = .02$; ANOVA, $F = 6.6$), bronchioles ($P = .003$; ANOVA, $F = 11.7$), and bronchi ($P = .002$; ANOVA, $F = 14.2$). The HPAI H5N1 group had significantly higher histological alveolar scores than did the seasonal H1N1 group ($P = .02$; Tukey test). The new H1N1 group had higher histological bronchiolar scores than

did the HPAI H5N1 group ($P = .003$; Tukey test) and the seasonal H1N1 group ($P = .02$; Tukey test). Both the new H1N1 group ($P = .002$; Tukey test) and the seasonal H1N1 group ($P = .02$; Tukey test) had significantly higher histological bronchial scores than did the HPAI H5N1 group.

On immunohistochemical analysis, influenza virus antigen expression was visible as diffuse-to-granular red staining, which usually was stronger in the nucleus than in the cytoplasm (Figure 3). Influenza virus antigen expression was closely associated with the presence of histological lesions at different levels of the lower respiratory tract. In the new H1N1 virus and HPAI H5N1 virus groups, influenza virus antigen expression was seen predominantly in type 1 and 2 pneumocytes (Figure 4*B* and 4*C*), alveolar macrophages, bronchiolar epithelial cells, and bronchial epithelial cells. In the seasonal H1N1 virus group, influenza virus antigen expression was seen in the same cell types but was rare at any level of the lower respiratory tract. Quantitative scoring showed that influenza virus antigen expression in the new H1N1 virus group was high at all 3 levels of the lower respiratory tract (alveoli, bronchioles, and bronchi) (Figure 5). This contrasted to the influenza virus antigen expression in the HPAI H5N1 virus group, which was highest in alveoli, and lower in bronchioles and bronchi; and with that in the seasonal H1N1 virus group, which was most prominent in bronchi, but low at all 3 levels. The immunohistochemical scores among the 3 virus groups differed significantly for the alveoli ($P < .001$; ANOVA, $F = 217$) and bronchi ($P = .01$; ANOVA, $F = 8.0$). The new H1N1 virus group had significantly higher immunohistochemical scores than the seasonal H1N1 virus group for alveoli ($P < .001$; Tukey test) and bronchi ($P = .01$; Tukey test), and significantly higher immunohistochemical bronchial scores than did the HPAI H5N1 virus group ($P = .03$; Tukey test). The HPAI H5N1 group had significantly higher immunohistochemical alveolar scores than did the seasonal H1N1 group ($P < .001$; Tukey test). None of the ferrets in the seasonal H1N1 virus and new H1N1 virus groups had significant lesions or influenza virus antigen expression in extrarespiratory tissues, which is a hallmark of infection of ferrets with HPAI H5N1 virus A/Indonesia/5/2005 [17, 18].

DISCUSSION

To cause a major pandemic, a newly emerging influenza virus needs to be not only efficiently transmissible among humans, but it also must be able to cause severe pneumonia. The former has been demonstrated by epidemiological analysis of its spread in the human population, which provided R_0 estimates in the range of 1.4–1.6, comparable with lower R_0 estimates obtained from previous pandemics [8]. This was corroborated by our previous study, which showed ferret-to-ferret transmission of new H1N1 virus was equally efficient as that of a seasonal H1N1 virus on intranasal inoculation [9, 19].

Here we show that the new H1N1 virus is not only efficiently transmissible but is also able to cause severe pneumonia in ferrets. With other factors held constant, the new H1N1 virus causes pneumonia that is intermediate in severity between that caused by seasonal H1N1 virus and that caused by HPAI H5N1 virus. The new H1N1 virus shares with HPAI H5N1 virus the ability to replicate well in epithelial cells in the lower respiratory tract and to cause diffuse alveolar damage. Our results correspond with those of Itoh et al [20], who found that ferrets inoculated intranasally with new H1N1 influenza virus showed more severe bronchopneumonia than those inoculated with a recent seasonal H1N1 virus.

Because the pattern of influenza virus attachment to the lower respiratory tract is similar for ferrets and humans [21], and because influenza-associated disease in ferrets resembles that in humans [22], a similar pattern of infection and associated disease by the new H1N1 virus may also be expected to occur in humans. This is corroborated by the clinical characterization of patients with confirmed fatal infection with new H1N1 virus, for whom acute respiratory distress syndrome was the most frequent diagnosis [6].

The warning from this study—with the caveat that no animal model is able to capture all aspects of the human disease—is the intrinsic ability of the new H1N1 virus to cause more severe pneumonia than seasonal H1N1 virus. This fact needs to be taken into account in ongoing pandemic preparedness planning. Furthermore, regular evaluation of the severity of the pneumonia caused by the new H1N1 virus in this ferret model will provide valuable information about possible changes in virulence as this newly emerged virus further adapts to its human host.

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