Journal of Microbiology (2013) Vol. 51, No. 6, pp. 866–871
DOI 10.1007/s12275-013-3428-7

DBA/2 Mouse as an Animal Model for Anti-influenza Drug Efficacy Evaluation

Jin Il Kim[†], Sehee Park[†], Sangmoo Lee[†], Ilseob Lee[†], Jun Heo, Min-Woong Hwang, Joon-Yong Bae, Donghwan Kim, Seok-Il Jang, Mee Sook Park, and Man-Seong Park[*]

Department of Microbiology, Center for Medical Science Research, College of Medicine, Hallym University, Chuncheon 200-702, Republic of Korea

(Received August 9, 2013 / Accepted September 3, 2013)

Influenza viruses are seasonally recurring human pathogens. Vaccines and antiviral drugs are available for influenza. However, the viruses, which often change themselves via antigenic drift and shift, demand constant efforts to update vaccine antigens every year and develop new agents with broad-spectrum antiviral efficacy. An animal model is critical for such efforts. While most human influenza viruses are unable to kill BALB/c mice, some strains have been shown to kill DBA/2 mice without prior adaptation. Therefore, in this study, we explored the feasibility of employing DBA/2 mice as a model in the development of anti-influenza drugs. Unlike the BALB/c strain, DBA/2 mice were highly susceptible and could be killed with a relatively low titer (50% DBA/2 lethal dose = $10^{2.83}$ plaque-forming units) of the A/Korea/01/2009 virus (2009 pandemic H1N1 virus). When treated with a neuraminidase inhibitor, oseltamivir phosphate, infected DBA/2 mice survived until 14 days post-infection. The reduced morbidity of the infected DBA/2 mice was also consistent with the oseltamivir treatment. Taking these data into consideration, we propose that the DBA/2 mouse is an excellent animal model to evaluate antiviral efficacy against influenza infection and can be further utilized for combination therapies or bioactivity models of existing and newly developed anti-influenza drugs.

Keywords: adaptation, animal model, antiviral, DBA/2 mouse, influenza virus

Introduction

Influenza viruses cause acute respiratory illnesses that range from mild to lethal (Neumann and Kawaoka, 2011). Prophylactic vaccinations or therapeutic antiviral drugs are used to address seasonally recurring influenza infections. However, due to the constantly changing nature of the virus (Palese and Shaw, 2007; Wright and Webster, 2007; Kim and Park, 2012b; Kim *et al*., 2013), vaccines must be constantly re-evaluated (Kim and Park, 2012a; Lee *et al*., 2013), and new drugs must be developed to cope with emerging resistant strains (Ison, 2011; Park *et al*., 2012). Hence, an animal model is critical for all such evaluations. Ferrets are deemed to be closest to humans in terms of the manifestation of influenza-like symptoms and transmission patterns (Bouvier and Lowen, 2010; Belser *et al*., 2011). Diverse indicators of symptomatic morbidity and mortality after highly pathogenic avian influenza (HPAI) H5N1 virus infection are also considered to be similar between ferrets and humans (Long *et al*., 2012). However, the high expenses involved in purchasing and keeping ferrets have deterred the use of these animals in large numbers, and an alternative model has been sought (Bouvier and Lowen, 2010). Guinea pigs may serve as an alternative to ferrets because they can represent aerosol transmission of human influenza viruses (Lowen *et al*., 2006). However, the lack of information regarding viral pathogenicity hinders the feasibility of guinea pigs in influenza virus research. Other than transmission studies, mice have been used successfully as animal models for influenza virus infections and for evaluations of factors affecting viral virulence (Maines *et al*., 2005; Salomon *et al*., 2006; Chen *et al*., 2008), vaccine efficacy (van der Laan *et al*., 2008), and antiviral drug efficacy (Sidwell *et al*., 2007; Yun *et al*., 2008; Kiso *et al*., 2010). However, not all influenza viruses are lethal to certain mice, necessitating the use of a cumbersome viral adaptation process to enhance lethality in the mice (Bouvier and Lowen, 2010).

The DBA/2 mouse garnered attention due to its much higher susceptibility to H5N1 virus infection than C56BL/6 mice (Boon *et al*., 2009). Subsequently, a variety of influenza viruses, including influenza B viruses, were shown to be lethal to DBA/2 mice without prior adaptation (Boon *et al*., 2010; Pica *et al*., 2011). Adaptation of the virus in mice, which transforms the virus to be lethal to mice, was deemed necessary to clearly minimize the possibility of false negativity (Sidwell *et al*., 1995) between the control and experimental conditions in virus infection experiments. However, evaluation of antiviral drug efficacy with a mouse-adapted virus *in vivo* can be complicated by genetic changes in the virus genome during the mouse adaptation process (Sakabe *et al*., 2011; Zhu *et al*., 2012). Thus, DBA/2 mice, which do not require prior adaptation of most influenza viruses, have been actively utilized in studies on the functions of influenza viral proteins (Hai *et al*., 2010), potentials of antibody therapy (Boon *et al*., 2010; Kashyap *et al*., 2010), and vaccine efficacy (Solorzano *et al*., 2010; Dengler *et al*., 2012). Using an un-

[†]These authors contributed equally to this work.
*For correspondence. E-mail: manseong.park@gmail.com; Tel.: +82-33-248-2632; Fax: +82-33-252-2843

adapted virus is critically important for testing the efficacy of anti-influenza drugs against human-infecting viruses, particularly during the drug development stage, as the antiviral effect of the drug should not be against the mouse-adapted virus but ultimately against the original human-infecting virus. However, there have been no reports on the use of DBA/2 mice in antiviral efficacy studies. Therefore, we evaluated the DBA/2 mouse as an animal model for testing efficacies of anti-influenza drugs *in vivo*. Here, as a proof of principle, we present the effect of a globally licensed anti-influenza drug, oseltamivir phosphate, on the replication and pathogenicity of a 2009 pandemic H1N1 (pH1N1) virus in infected DBA/2 mice.

Materials and Methods

Virus and cells

The pH1N1 virus, A/Korea/01/2009 (K/09), was provided by the Korea Centers for Disease Control and Prevention (KCDC; Osong, Korea). The virus was propagated in 10-day-old embryonated chicken eggs. To determine virus titer, a standard plaque assay was performed in Madin-Darby canine kidney (MDCK) cells, which were obtained from the American Type Culture Collection (ATCC, USA). MDCK cells were maintained with Eagle's minimum essential medium (Lonza, Switzerland) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA), 100 U/ml of penicillin, and 100 μg/ml streptomycin (Gibco, USA) at 37°C in a 5% CO_2 incubator.

Animal experiments

BALB/c mice (female, 5 weeks old; NaraBiotech, Korea) and DBA/2 mice (female, 5 weeks old; Japan SLC, Inc., Japan) were used in this study, and all of the animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Animal, Plant, and Fisheries Quarantine and Inspection Agency of Korea. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hallym University (permit number: Hallym 2013-11).

Determination of 50% mouse lethal dose

To determine 50% mouse lethal dose (MLD_{50}) titers of mice and their body weight changes, five BALB/c and five DBA/2 mice per group were anesthetized and intranasally inoculated with 10^3, 10^4, 10^5, or 10^6 plaque-forming units (PFU) (BALB/c mice) and 10^0, 10^1,10^2, 10^3, 10^4, or 10^5 PFU (DBA/2 mice) of the K/09 virus. Body weight changes and survival rates were monitored daily until 14 days post-infection (dpi). The percentage of body weight loss was determined by comparing the body weight of each mouse in the experiment to its initial body weight. Mice that experienced more than 25% weight loss were considered experimentally dead and humanely euthanized. The MLD_{50} was determined using the Reed and Muench method (Reed and Muench, 1938).

Virus titers in the lungs of infected mice

DBA/2 mice were infected intranasally with 10 MLD_{50} (equivalent to $10^{3.83}$ PFU) of the K/09 virus. Lung samples of three infected mice were collected at each indicated dpi and homogenized, and the virus titers were then determined by a standard plaque assay in MDCK cells.

Anti-influenza drug efficacy test

An influenza neuraminidase inhibitor, oseltamivir phosphate (oseltamivir; Toronto Research Chemicals, Canada), was used as a test anti-influenza chemical. Six DBA/2 mice per group were anesthetized and intranasally infected with 10 MLD_{50} of the K/09 virus. Oseltamivir (90 mg/kg/day) or PBS (mock group) was orally administered to the infected mice twice daily for eight days, beginning two days before

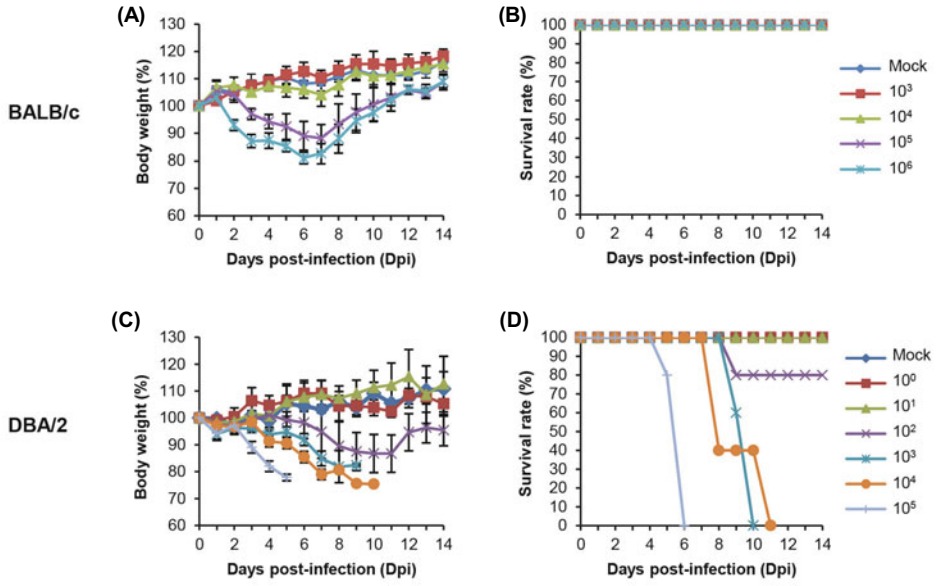

Fig. 1. Body weight changes and survival rates of BALB/c or DBA/2 mice infected with the A/Korea/01/2009 virus. (A-D) To analyze the pathogenicity of the K/09 virus, five BALB/c (A-B) and DBA/2 (C-D) mice (female, five weeks old) per group were anesthetized and intranasally infected with the virus. Body weight changes and survival rates were recorded until 14 dpi. Mean percentage body weight losses± standard deviations (SD) were plotted (A and C). Mice that experienced more than 25% weight loss were considered experimentally dead (B and D). As a control, mice in the mock group were infected with PBS.

Table 1. Lethal doses of A/Korea/01/2009 virus in BALB/c and DBA/2 mice.

Virus	MLD_{50}[a] in the indicated mouse model	
	BALB/c	DBA/2
K/09	$>10^6$	$10^{2.83}$

[a] The MLD_{50} was determined by the Reed and Muench method.

infection. Body weight changes and survival rates were monitored daily until 14 dpi. To evaluate the antiviral efficacy against virus replication in the lungs of mice, the same procedures above (see 'Materials and Methods' section 'Virus titers in the lungs of infected mice') were performed after the oseltamivir treatment (90 mg/kg/day, twice daily until 3 or 6 dpi).

Statistical analysis

The statistical significance of the differences in virus titers in the lungs of infected mice between PBS- and oseltamivir-treated mice was assessed using Student's *t*-test.

Results and Discussion

Pathogenicity of the pH1N1 K/09 virus in BALB/c versus DBA/2 mice

We first determined the pathogenicity of K/09 virus infection in BALB/c and DBA/2 mice. Because body weight loss has been shown to be a good early indicator symptom of eventual mortality in influenza virus-infected BALB/c mice (Trammell and Toth, 2011), we measured body weight changes as a marker of morbidity. The K/09 virus was non-lethal to BALB/c mice, even with an intranasal inoculation of 10^6 PFU (Fig. 1A and 1B). The maximum body weight loss was observed at 6 dpi but reached less than 20%, and the infected BALB/c mice eventually recovered their initial body weights after 10 dpi (Fig. 1A). In contrast, DBA/2 mice succumbed to the virus and started to die at 5 dpi (Fig. 1C and 1D). Fatality was inevitable in DBA/2 mice infected with as low as 10^2 PFU, and the MLD_{50} value of the K/09 virus was determined to be $10^{2.83}$ PFU (Table 1).

As reported previously (Itoh *et al.*, 2009; Belser *et al.*, 2010; Kwon *et al.*, 2010; Manicassamy *et al.*, 2010), the K/09 virus appeared to be less pathogenic in BALB/c mice compared with other pH1N1 viruses (Fig. 1A and 1B). However, DBA/2 mice were highly susceptible to the K/09 virus, although they recovered from exposure to a non-lethal dose of the K/09 virus (Fig. 1C and 1D), similar to BALB/c mice. This result suggests that the high susceptibility of DBA/2 mice to influenza viruses may not be due to impaired immune responses against influenza viruses (Dengler *et al.*, 2012) and that the DBA/2 mouse model may be otherwise comparable with other mouse models.

DBA/2 mice as an animal model for anti-influenza drug efficacy testing

Based on the characteristics of K/09 virus infection in DBA/2 mice, as a proof of principle, we sought to evaluate the globally licensed influenza neuraminidase inhibitor drug, oseltamivir, in the protection of DBA/2 mice against K/09 virus infection. Oseltamivir has been previously tested alone or in combination with other drugs in several other mouse systems using mouse-adapted viruses (Mendel *et al.*, 1998; Nguyen *et al.*, 2012) and using non-lethal mouse infection models (Wong *et al.*, 2011). The efficacy of oseltamivir treatment in ferrets against pH1N1 Ca/04 virus infection has also been measured by diverse non-lethal indicators (Govorkova *et al.*, 2011).

We used oseltamivir to determine the feasibility of employing DBA/2 mice in testing the *in vivo* efficacies of anti-

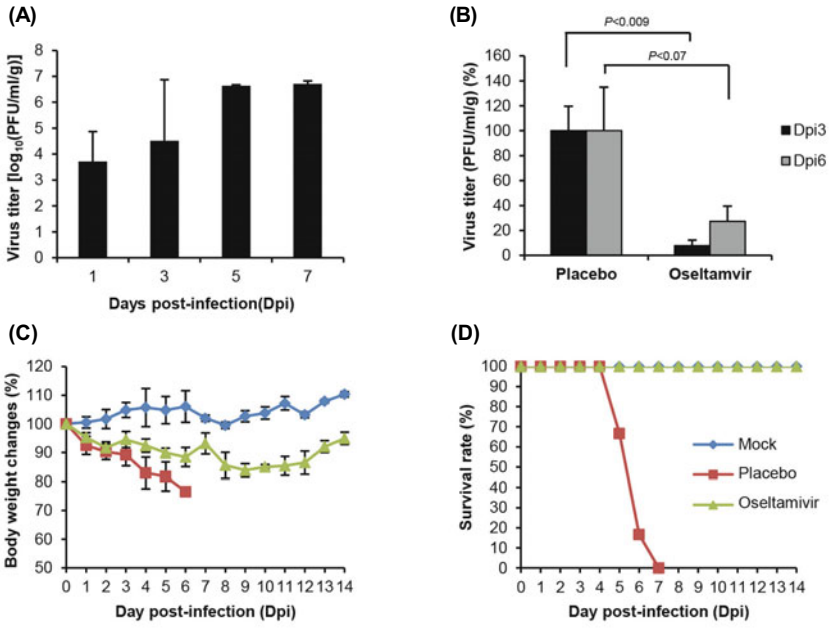

Fig. 2. Viral replication and the therapeutic efficacy of oseltamivir in DBA/2 mice. (A) To determine K/09 virus replication characteristics in the lungs of DBA/2 mice after high-dose infection, three mice per group were intranasally infected with 10 MLD_{50}. Lung viral titers at 1, 3, 5, and 7 dpi were plotted as the mean $\log_{10}$ PFU±SD. (B-D) To determine the efficacy of oseltamivir against high-dose K/09 virus infection in DBA/2 mice, three mice per group for lung titers (B) and six mice per group for body weight loss and survival rate determinations (C-D) were anesthetized and infected intranasally with 10 MLD_{50} of the K/09 virus. Administration of oseltamivir 90 mg/kg/day or PBS to the mice started from two days before infection and continued until 6 dpi. Lung titers at 3 and 6 dpi were plotted as percentages relative to an average of the placebo group±SD (B). Body weight changes were recorded daily until 14 dpi, and mean body weight losses±SD were plotted (C). Mice that experienced more than 25% body weight loss were considered experimentally dead (D). As a control, mice in the mock group were infected with PBS.

viral drugs in the future. Initially, we determined the K/09 virus replication profile in the lungs of DBA/2 mice after a high-dose infection of 10 MLD_{50} (Fig. 2A). Lung titers from the infected mice reached a high titer plateau ($10^{6.6}$ PFU/ml/g) at 5 dpi, which was sustained until 7 dpi. Because there was no reported precedence of using DBA/2 mice in antiviral drug studies, we attempted to demonstrate the protective efficacy of an antiviral drug in DBA/2 mice infected with a high dose (10 MLD_{50}) of the pH1N1 virus. A previous study reported that BALB/c mice after lethal infection with mouse-adapted Ca/04 virus could be treated with 50 mg/kg/day oseltamivir (Zarogiannis *et al*., 2012). However, another study using ferrets suggested that higher virulence and a higher viral input dose require a higher amount of oseltamivir administration for therapeutic outcomes (Govorkova *et al*., 2007). Based on those studies, we decided to try administering oseltamivir 90 mg/kg/day to DBA/2 mice from two days prior to K/09 virus infection. The virus titers in the lungs of infected mice were 14- and 3.7-fold different at 3 dpi and 6 dpi, respectively, between oseltamivir- and PBS-treated placebo groups (Fig. 2B). The effect of oseltamivir under this experimental condition was demonstrated by 100% survival of the infected DBA/2 mice compared with 0% of the mice in the placebo group (Fig. 2C and 2D).

Symptoms of influenza infection observed in humans, such as sneezing and lethargy, have been observed in ferrets infected with high doses of a human-infecting HPAI H5N1 virus (Long *et al*., 2012). Mice non-lethally infected with influenza virus also began to show signs of sickness, measured by locomotor activity, from 3 dpi (Majde *et al*., 2010). The difference in the viral titers in the lungs of the infected DBA/2 mice at 3 dpi (Fig. 2B) might have been sufficient to determine whether the mice would succumb to death or eventually recover. In ferrets with lethal infections with the HPAI H5N1 virus, administration of the same amount of oseltamivir was shown to result in either eventual survival or death of all of the ferrets according to the time of oseltamivir administration (4 h or 24 h post-infection, respectively) (Govorkova *et al*., 2007). In addition to the results in Fig. 2, these data are in agreement with the reported benefits of early intervention with oseltamivir treatment after the onset of influenza-like symptoms in patients (Aoki *et al*., 2003). Because DBA/2 mice were as competent as C57BL/6 mice in immune responses against influenza (Dengler *et al*., 2012), the small difference in the lung titers at 6 dpi might suggest a start of the contribution of the immune responses in DBA/2 mice to maintaining viral titers at a certain level.

Our data also showed that the DBA/2 mouse could be a good model for measuring the effects of an anti-influenza drug against the morbidity caused by influenza virus infection. Despite lethal infection with a 10 MLD_{50} dose of the virus, 90 mg/kg/day oseltamivir notably reduced body weight loss in infected DBA/2 mice (Fig. 2C). At 6 dpi, when all placebo mice reached experimental death by losing more than 25% of their body weight, oseltamivir treatment protected mice from excessive weight loss and severe viral replication in the lungs (Figs. 2B and 2C).

To verify whether 25% weight loss indeed eventually leads to death, we observed body weight changes of three mice in the PBS-treatment group because it was reported that body weight loss alone might not be an early indicator of eventual death for DBA/2 mice (Trammell and Toth, 2011). All three mice actually died at 9 dpi (data not shown). According to our observation, body weight loss of more than 25% led to the eventual death of the animal. Therefore, the efficacy of an anti-influenza drug against pH1N1 virus could clearly be demonstrated in DBA/2 mice by the simple indicator of body weight loss.

Perspective of the DBA/2 mouse model

Although ferrets are believed to best reflect humans among influenza virus infection models (Bouvier and Lowen, 2010; Belser *et al*., 2011), a mouse model could be used more widely and effectively. *In vivo* drug tests using animal models assume that the bioactivities of the drugs in the animals can be extrapolated to humans. There is no evidence that ferrets are better than mice in that regard. In fact, while the bioavailability of oseltamivir was higher than 90% in humans (Wattanagoon *et al*., 2009), it was 30% and 11% in mice and ferrets, respectively (Li *et al*., 1998). Therefore, an animal model should represent only certain aspects of human infection.

In terms of anti-influenza drug studies, *in vivo* drug efficacy has been associated with reduction of viral titers in test animals (Govorkova *et al*., 2007). If there is a clear measure, such as viral titers or the viral RNA copies, to differentiate the experimental from the control conditions, a non-lethal mouse model with mouse-adapted or non-adapted viruses or any other non-lethal animal model could be used without concerns of false negativity (Sidwell *et al*., 1995). However, occasionally, the differences in viral titers are unclear (Govorkova *et al*., 2011), and it is difficult to determine if the time point and the degree of difference alone can serve as critical indicators of drug efficacy. We observed a 14-fold difference in the viral lung titers between oseltamivir- and PBS-treated groups (Fig. 2B). However, without information on survival rates (Fig. 2D), it would have been difficult for us to evaluate significant differences as beneficial effects of the drug in animals.

From the survival rate data, we conclude that DBA/2 mice can be effectively used as a non-adapted influenza virus infection model in which the effects of an anti-influenza drug on morbidity and mortality can be clearly determined by body weight measurement. DBA/2 mice may be especially useful for evaluating combination therapies or testing newly screened anti-influenza drugs for *in vivo* bioactivity against multiple influenza virus strains.

Acknowledgements

This study was supported by grants from the Korea Healthcare Technology R&D Project of the Ministry of Health & Welfare (Grant No. A103001), the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (Grant No. 2007-0052178), and the Hallym University Specialization Fund (HRF-S-41).

References

Aoki, F.Y., Macleod, M.D., Paggiaro, P., Carewicz, O., El Sawy, A., Wat, C., Griffiths, M., Waalberg, E., Ward, P., and Group, I.S. 2003. Early administration of oral oseltamivir increases the benefits of influenza treatment. *J. Antimicrob. Chemother.* **51**, 123–129.

Belser, J.A., Katz, J.M., and Tumpey, T.M. 2011. The ferret as a model organism to study influenza A virus infection. *Dis. Model. Mech.* **4**, 575–579.

Belser, J.A., Wadford, D.A., Pappas, C., Gustin, K.M., Maines, T.R., Pearce, M.B., Zeng, H., Swayne, D.E., Pantin-Jackwood, M., Katz, J.M., and *et al.* 2010. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. *J. Virol.* **84**, 4194–4203.

Boon, A.C., deBeauchamp, J., Hollmann, A., Luke, J., Kotb, M., Rowe, S., Finkelstein, D., Neale, G., Lu, L., Williams, R.W., and *et al.* 2009. Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. *J. Virol.* **83**, 10417–10426.

Boon, A.C., deBeauchamp, J., Krauss, S., Rubrum, A., Webb, A.D., Webster, R.G., McElhaney, J., and Webby, R.J. 2010. Cross-reactive neutralizing antibodies directed against pandemic H1N1 2009 virus are protective in a highly sensitive DBA/2 mouse influenza model. *J. Virol.* **84**, 7662–7667.

Bouvier, N.M. and Lowen, A.C. 2010. Animal models for influenza virus pathogenesis and transmission. *Viruses* **2**, 1530–1563.

Chen, L.M., Davis, C.T., Zhou, H., Cox, N.J., and Donis, R.O. 2008. Genetic compatibility and virulence of reassortants derived from contemporary avian H5N1 and human H3N2 influenza A viruses. *PLoS Pathog.* **4**, e1000072.

Dengler, L., May, M., Wilk, E., Bahgat, M.M., and Schughart, K. 2012. Immunization with live virus vaccine protects highly susceptible DBA/2J mice from lethal influenza A H1N1 infection. *Virol. J.* **9**, 212.

Govorkova, E.A., Ilyushina, N.A., Boltz, D.A., Douglas, A., Yilmaz, N., and Webster, R.G. 2007. Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza virus. *Antimicrob. Agents Chemother.* **51**, 1414–1424.

Govorkova, E.A., Marathe, B.M., Prevost, A., Rehg, J.E., and Webster, R.G. 2011. Assessment of the efficacy of the neuraminidase inhibitor oseltamivir against 2009 pandemic H1N1 influenza virus in ferrets. *Antiviral. Res.* **91**, 81–88.

Hai, R., Schmolke, M., Varga, Z.T., Manicassamy, B., Wang, T.T., Belser, J.A., Pearce, M.B., Garcia-Sastre, A., Tumpey, T.M., and Palese, P. 2010. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. *J. Virol.* **84**, 4442–4450.

Ison, M.G. 2011. Antivirals and resistance: influenza virus. *Curr. Opin. Virol.* **1**, 563–573.

Itoh, Y., Shinya, K., Kiso, M., Watanabe, T., Sakoda, Y., Hatta, M., Muramoto, Y., Tamura, D., Sakai-Tagawa, Y., Noda, T., and *et al.* 2009. *In vitro* and *in vivo* characterization of new swine-origin H1N1 influenza viruses. *Nature* **460**, 1021–1025.

Kashyap, A.K., Steel, J., Rubrum, A., Estelles, A., Briante, R., Ilyushina, N.A., Xu, L., Swale, R.E., Faynboym, A.M., Foreman, P.K., and *et al.* 2010. Protection from the 2009 H1N1 pandemic influenza by an antibody from combinatorial survivor-based libraries. *PLoS Pathog.* **6**, e1000990.

Kim, J.I., Lee, I., Park, S., Hwang, M.W., Bae, J.Y., Lee, S., Heo, J., Park, M.S., Garcia-Sastre, A., and Park, M.S. 2013. Genetic requirement for hemagglutinin glycosylation and its implications for influenza A H1N1 virus evolution. *J. Virol.* **87**, 7539–7549.

Kim, J.I. and Park, M.S. 2012a. An universal approach to getting ahead for influenza B vaccines. *J. Bacteriol. Virol.* **42**, 363–367.

Kim, J.I. and Park, M.S. 2012b. N-linked glycosylation in the hemagglutinin of influenza A viruses. *Yonsei Med. J.* **53**, 886–893.

Kiso, M., Takahashi, K., Sakai-Tagawa, Y., Shinya, K., Sakabe, S., Le, Q.M., Ozawa, M., Furuta, Y., and Kawaoka, Y. 2010. T-705 (favipiravir) activity against lethal H5N1 influenza A viruses. *Proc. Natl. Acad. Sci. USA* **107**, 882–887.

Kwon, D., Shin, K., Kim, S., Ha, Y., Choi, J.H., Yang, J.S., Lee, J.Y., Chae, C., Oh, H.B., and Kang, C. 2010. Replication and pathogenesis of the pandemic (H1N1) 2009 influenza virus in mammalian models. *J. Microbiol.* **48**, 657–662.

Lee, I., Kim, J.I., and Park, M.S. 2013. Cell-culture-based influenza vaccines as alternatives to egg-based vaccines. *J. Bacteriol. Virol.* **43**, 9–17.

Li, W., Escarpe, P.A., Eisenberg, E.J., Cundy, K.C., Sweet, C., Jakeman, K.J., Merson, J., Lew, W., Williams, M., Zhang, L., and *et al.* 1998. Identification of GS 4104 as an orally bioavailable prodrug of the influenza virus neuraminidase inhibitor GS 4071. *Antimicrob. Agents Chemother.* **42**, 647–653.

Long, J.P., Vela, E.M., Stark, G.V., Jones, K.J., Miller, S.T., and Bigger, J.E. 2012. Early indicators of disease in ferrets infected with a high dose of avian influenza H5N1. *Sci. Rep.* **2**, 972.

Lowen, A.C., Mubareka, S., Tumpey, T.M., Garcia-Sastre, A., and Palese, P. 2006. The guinea pig as a transmission model for human influenza viruses. *Proc. Natl. Acad. Sci. USA* **103**, 9988–9992.

Maines, T.R., Lu, X.H., Erb, S.M., Edwards, L., Guarner, J., Greer, P.W., Nguyen, D.C., Szretter, K.J., Chen, L.M., Thawatsupha, P., and *et al.* 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J. Virol.* **79**, 11788–11800.

Majde, J.A., Kapas, L., Bohnet, S.G., De, A., and Krueger, J.M. 2010. Attenuation of the influenza virus sickness behavior in mice deficient in Toll-like receptor 3. *Brain Behav. Immun.* **24**, 306–315.

Manicassamy, B., Medina, R.A., Hai, R., Tsibane, T., Stertz, S., Nistal-Villan, E., Palese, P., Basler, C.F., and Garcia-Sastre, A. 2010. Protection of mice against lethal challenge with 2009 H1N1 influenza A virus by 1918-like and classical swine H1N1 based vaccines. *PLoS Pathog* **6**, e1000745.

Mendel, D.B., Tai, C.Y., Escarpe, P.A., Li, W., Sidwell, R.W., Huffman, J.H., Sweet, C., Jakeman, K.J., Merson, J., Lacy, S.A., and *et al.* 1998. Oral administration of a prodrug of the influenza virus neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. *Antimicrob. Agents Chemother.* **42**, 640–646.

Neumann, G. and Kawaoka, Y. 2011. The first influenza pandemic of the new millennium. *Influenza Other Rrespi. Viruses* **5**, 157–166.

Nguyen, J.T., Smee, D.F., Barnard, D.L., Julander, J.G., Gross, M., de Jong, M.D., and Went, G.T. 2012. Efficacy of combined therapy with amantadine, oseltamivir, and ribavirin *in vivo* against susceptible and amantadine-resistant influenza A viruses. *PLoS One* **7**, e31006.

Palese, P. and Shaw, M.L. 2007. *Orthomyxoviridae*: The viruses and their replication. *In* Knipe, D.M. and Howley, P.M. (eds.), Fields virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA.

Park, S., Kim, J.I., and Park, M.S. 2012. Antiviral agents against influenza viruses. *J. Bacteriol. Virol.* **42**, 284–293.

Pica, N., Iyer, A., Ramos, I., Bouvier, N.M., Fernandez-Sesma, A., Garcia-Sastre, A., Lowen, A.C., Palese, P., and Steel, J. 2011. The DBA.2 mouse is susceptible to disease following infection with a broad, but limited, range of influenza A and B viruses. *J. Virol.* **85**, 12825–12829.

Reed, L.J. and Muench, H. 1938. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 5.

Sakabe, S., Ozawa, M., Takano, R., Iwastuki-Horimoto, K., and Kawaoka, Y. 2011. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. *Virus Res.* **158**, 124–129.

Salomon, R., Franks, J., Govorkova, E.A., Ilyushina, N.A., Yen, H.L., Hulse-Post, D.J., Humberd, J., Trichet, M., Rehg, J.E., Webby, R.J., and *et al.* 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *J. Exp. Med.* **203**, 689–697.

Sidwell, R.W., Bailey, K.W., Wong, M.H., and Huffman, J.H. 1995. *In vitro* and *in vivo* sensitivity of a non-mouse-adapted influenza A (Beijing) virus infection to amantadine and ribavirin. *Chemotherapy* **41**, 455–461.

Sidwell, R.W., Barnard, D.L., Day, C.W., Smee, D.F., Bailey, K.W., Wong, M.H., Morrey, J.D., and Furuta, Y. 2007. Efficacy of orally administered T-705 on lethal avian influenza A (H5N1) virus infections in mice. *Antimicrob. Agents Chemother.* **51**, 845–851.

Solorzano, A., Ye, J., and Perez, D.R. 2010. Alternative live-attenuated influenza vaccines based on modifications in the polymerase genes protect against epidemic and pandemic flu. *J. Virol.* **84**, 4587–4596.

Trammell, R.A. and Toth, L.A. 2011. Markers for predicting death as an outcome for mice used in infectious disease research. *Comp. Med.* **61**, 492–498.

van der Laan, J.W., Herberts, C., Lambkin-Williams, R., Boyers, A., Mann, A.J., and Oxford, J. 2008. Animal models in influenza vaccine testing. *Expert. Rev. Vaccines* **7**, 783–793.

Wattanagoon, Y., Stepniewska, K., Lindegardh, N., Pukrittayakamee, S., Silachamroon, U., Piyaphanee, W., Singtoroj, T., Hanpithakpong, W., Davies, G., Tarning, J., and *et al.* 2009. Pharmacokinetics of high-dose oseltamivir in healthy volunteers. *Antimicrob. Agents Chemother.* **53**, 945–952.

Wong, Z.X., Jones, J.E., Anderson, G.P., and Gualano, R.C. 2011. Oseltamivir treatment of mice before or after mild influenza infection reduced cellular and cytokine inflammation in the lung. *Influenza Other Respi. Viruses* **5**, 343–350.

Wright, P.F. and Webster, R.G. 2007. Orthomyxoviruses. *In* Knipe, D.M. and Howley, P.M. (eds.), Fields virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA.

Yun, N.E., Linde, N.S., Zacks, M.A., Barr, I.G., Hurt, A.C., Smith, J.N., Dziuba, N., Holbrook, M.R., Zhang, L., Kilpatrick, J.M., and *et al.* 2008. Injectable peramivir mitigates disease and promotes survival in ferrets and mice infected with the highly virulent influenza virus, A/Vietnam/1203/04 (H5N1). *Virology* **374**, 198–209.

Zarogiannis, S.G., Noah, J.W., Jurkuvenaite, A., Steele, C., Matalon, S., and Noah, D.L. 2012. Comparison of ribavirin and oseltamivir in reducing mortality and lung injury in mice infected with mouse adapted A/California/04/2009 (H1N1). *Life Sci.* **90**, 440–445.

Zhu, W., Zhu, Y., Qin, K., Yu, Z., Gao, R., Yu, H., Zhou, J., and Shu, Y. 2012. Mutations in polymerase genes enhanced the virulence of 2009 pandemic H1N1 influenza virus in mice. *PLoS One* **7**, e33383.