Published in final edited form as:

Vaccine. 2012 August 17; 30(38): 5603–5610. doi:10.1016/j.vaccine.2012.06.088.

Evaluation of replication, immunogenicity and protective efficacy of a live attenuated cold-adapted pandemic H1N1 influenza virus vaccine in non-human primates

Kobporn Boonnak¹, Myeisha Paskel¹, Yumiko Matsuoka¹, Leatrice Vogel¹, and Kanta Subbarao^{1,*}

¹Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, Maryland 20892

Abstract

We studied the replication of influenza A/California/07/09 (H1N1) wild type (CA09 *wt*) virus in two non-human primate species and used one of these models to evaluate the immunogenicity and protective efficacy of a live attenuated cold-adapted vaccine, which contains the hemagglutinin and neuraminidase from the H1N1 wild type virus and six internal protein gene segments of the A/Ann Arbor/6/60 cold-adapted (*ca*) master donor virus. We infected African green monkeys (AGMs) and rhesus macaques with 2×10^6 TCID₅₀ of CA09 *wt* and CA09 *ca* influenza viruses. The virus replicated in the upper respiratory tract of all animals but the titers in upper respiratory tract tissues of rhesus macaques were significantly higher than in AGMs (mean peak titers $10^{4.5}$ TCID₅₀/g and $10^{2.0}$ TCID₅₀/g on days 4 and 2 post-infection, respectively; $p < 0.01$). Virus replication was observed in the lungs of all rhesus macaques ($10^{2.0}$ – $10^{5.4}$ TCID₅₀/g) whereas only 2 out of 4 AGMs had virus recovered from the lungs ($10^{2.5}$ – $10^{3.5}$ TCID₅₀/g). The CA09 *ca* vaccine virus was attenuated and highly restricted in replication in both AGMs and rhesus macaques. We evaluated the immunogenicity and protective efficacy of the CA09 *ca* vaccine in rhesus macaques because CA09 *wt* virus replicated more efficiently in this species. One or two doses of vaccine were administered intranasally and intratracheally to rhesus macaques. For the two-dose group, the vaccine was administered 4-weeks apart. Immunogenicity was assessed by measuring hemagglutination-inhibiting (HAI) antibodies in the serum and specific IgA antibodies to CA09 *wt* virus in the nasal wash. One or two doses of the vaccine elicited a significant rise in HAI titers (range 40–320). Two doses of CA09 *ca* elicited higher pH1N1-specific IgA titers than in the mock-immunized group ($p < 0.01$). Vaccine efficacy was assessed by comparing titers of CA09 *wt* challenge virus in the respiratory tract of mock immunized and CA09 *ca* vaccinated monkeys. Significantly lower virus titers were observed in the lungs of vaccinated animals than mock-immunized animals ($p = 0.01$). Our results demonstrate that AGMs and rhesus macaques support the replication of pandemic H1N1 influenza virus to different degrees and a cold-adapted pH1N1 vaccine elicits protective immunity against pH1N1 virus infection in rhesus macaques.

Keywords

Pandemic H1N1; Non-human primate; Influenza vaccine

Corresponding author: Kanta Subbarao, Mailing address: Emerging Respiratory Viruses Section, Laboratory of Infectious Diseases, NIAID, NIH, Bldg.33, Room 3E13C.1, 33 North Drive, MSC 3203, Bethesda, MD 20892-3203., Phone: (301) 451-3839, Fax: (301) 480-4749, KSUBBARAO@niaid.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The 2009 swine-origin H1N1 influenza virus caused the first influenza pandemic of the 21st century [1–3]. We had previously studied the pathogenesis of pH1N1 (A/California/7/2009 H1N1, CA09*wt*) [4] and evaluated the efficacy and immunogenicity of a live attenuated cold adapted (*ca*) reassortant vaccine, A/California/7/2009 (CA09*ca*), in mice and ferrets [5]. Although small animal models such as mice and ferrets are often used to study the pathogenesis of influenza viruses, these animals do not fully mirror the response to live attenuated influenza vaccines in humans [6, 7]. Non-human primate models have been used less extensively in influenza vaccine research. New World [squirrel monkeys (*Saimiri sciureus*), owl monkeys (*Aotus trivirgatus*) and cebus monkeys (*Cebus apella* and *Cebus albifrons*)] and Old World species of monkeys [pigtailed macaques (*Macaca nemestrina*) and Cynomolgus macaques (*Macaca fascicularis*)] have been evaluated as models for influenza virus infection and a broad spectrum of clinical signs and degree of influenza virus replication in the respiratory tract were observed among the different species [8–13]. Squirrel monkeys developed mild upper respiratory illness following intratracheally inoculation of influenza A/Udorn/72 (H3N2), A/Alaska/77 (H3N2) and A/Hong Kong/77 (H1N1) viruses whereas no clinical signs were observed in owl and cebus monkeys infected with these viruses [9]. Among the Old World monkeys, pigtailed macaques (*Macaca nemestrina*) exhibited clinical signs of infection such as weight loss, nasal discharge and moderate fever following inoculation of A/Texas/91 (H1N1) by multiple routes [10]. Cynomolgus macaques (*Macaca fascicularis*) have been used to study highly pathogenic H5N1 viruses [14, 15], the 1918 pandemic virus [16]. Thus far, three published studies have investigated the pathogenic potential of the pH1N1 virus in cynomolgus macaques. pH1N1 virus-infected cynomolgus macaques showed clinical disease ranging from mild to severe pneumonia that was more severe than the mild illness associated with seasonal H1N1 virus [13]. The pH1N1 virus replicated efficiently in the lungs and other organs of infected cynomolgus macaques whereas seasonal influenza viruses were typically limited in their replicative ability to the lungs of infected primates [11–13]. Pathological examination revealed that pH1N1 infection caused lung lesions that were intermediate in severity between those observed following infection with highly pathogenic avian H5N1 and seasonal H1N1 viruses. Although pathogenesis of the pH1N1 virus has been studied in non-human primates, there are no reports of the evaluation of the pH1N1 virus vaccine in this animal model.

We designed a study to evaluate the immunogenicity and protective efficacy of the live attenuated CA09*ca* pH1N1 vaccine in non-human primates. We found that replication of the CA09*wt* virus was different in two species of non-human primates; rhesus macaques supported replication of CA09*wt* and CA09*ca* viruses better than African green monkeys. In both species, the CA09*wt* virus replicated in the upper and lower respiratory tract, whereas replication of the CA09*ca* vaccine strain was severely restricted in the lower respiratory tract. We studied the immunogenicity and protective efficacy of the CA09*ca* virus in rhesus macaques, and found that vaccination with either 1 or 2 doses of vaccine elicited a protective antibody titer and conferred protection against challenge with the CA09*wt* virus.

2. Materials and Methods

2.1 Viruses

The wild type (*wt*) pandemic H1N1 virus, A/California/7/2009 (CA09*wt*), was kindly provided by the Influenza Division, Centers for Disease Control and Prevention (CDC). The CA09*wt* virus was propagated in the allantoic cavity of 9-to 11-day-old embryonated specific pathogen-free hen's eggs. The titer of the virus was determined in Madin-Darby Canine Kidney (MDCK) cells. Allantoic fluid from passage 4 was used in this study. There

are 2 amino acid differences (N125D and Q223R) between the HA proteins of the CA09 *wt* virus used in this study and those available in Genbank (FJ969540.1). The live attenuated cold-adapted (*ca*) reassortant virus, A/California/7/2009 (CA09 *ca*) H1N1 virus, was generated by reverse genetics [17]. In brief, the H1 HA and N1 NA of the reassortant pandemic H1N1 *ca* vaccine virus were derived from the CA09 *wt* virus, and the internal gene segments were derived from A/Ann Arbor/6/60 *ca* (AA *ca*; H2N2) master donor virus. The CA09 *ca* virus was propagated in embryonated eggs and passage 3 was used in this study. The CA09 *ca* virus contains two additional amino acid changes (K119E and A186D) in the HA protein that enhanced vaccine virus yield in eggs, without affecting vaccine antigenicity and immunogenicity in ferrets [17].

2.2 Non-human primates

Studies were carried out in 25 approximately 3- to 4-year-old male or female African green monkeys (AGM; *Chlorocebus aethiops*) and 37 rhesus macaques (*Macaca mulatta*). The monkeys were examined each day for evidence of clinical illness such as body temperature, pulse, appetite, behavior, stool, respiratory symptoms, and ocular signs. All animal experiments were done at the NIH, in compliance with the guidelines of the NIAID/NIH Animal Care and Use Committee.

2.3 Inoculation and immunization

For inoculation and immunization, the monkeys were anesthetized by intramuscular injection (I.M.) with Ketamine HCl (10mg/kg) and the *wt* or vaccine virus was delivered intranasally (I.N.) and intratracheally (I.T.) with 1 ml by each route containing 1×10^6 TCID₅₀ of the virus. Four animals in each group received 1 or 2 doses of vaccine. Additionally, two monkeys in each group were mock immunized. Sera were collected on days 28 and 56 following immunization.

2.4 Serological evaluation

2.4.1 Hemagglutination inhibition (HAI) assay—The HAI assay was performed as previously described [18, 19]. Briefly, ferret sera treated with receptor destroying enzyme (RDE, SEIKEN, Campbell, CA) were 2-fold serially diluted in 96-well V-bottom plates starting at a dilution of 1:10, and 4 HA units of virus was added. Control wells received PBS alone or PBS with virus in the absence of antibody. Virus and sera were incubated together for 30 min at room temperature. Next, 50 μ l of a 0.5% (vol/vol) suspension of turkey erythrocytes was added. The antibody, virus, and erythrocytes were gently mixed, and the results were recorded after incubating for 45–60 min at room temperature. HAI titers were recorded as the inverse of the highest antibody dilution that inhibited hemagglutination.

2.4.2 Specific IgA assay—ELISA was performed as previously described [20]. Briefly, plates were coated with β -propiolactone-inactivated CA09 *wt* virus at 1,000 hemagglutinating units (HAU)/ml (50 μ l/well) or baculovirus-expressed recombinant HA protein from the pH1N1 virus (NR15258) obtained through the National Institutes of Health Biodefense and Emerging Infectious Diseases Repository and used at 1 μ g/ml (50 μ l per well). Nasal wash samples were incubated on the plates at 4°C overnight. Bound antibodies were detected with biotinylated goat anti-monkey IgA (α -chain) and HRP conjugated streptavidin (Dako, Carpinteria, CA). Wells with an OD of >0.2 at 450 nm were considered positive.

2.5 Replication studies

To determine the replication kinetics of CA09 *wt* and CA09 *ca* viruses, groups of animals were inoculated with 2×10^6 TCID₅₀ of the respective viruses. On days 2, 4 and 7 after

inoculation, animals were euthanized and eight portions of lung (right caudal distal and proximal, left caudal distal and proximal, right cranial distal and proximal, left cranial distal and proximal), nasal turbinates, and intestines (duodenum, ileocecal valve, jejunum-ileal loop and descending colon) were harvested from each group. Organs were homogenized in L-15 medium containing antibiotic-antimycotic (Invitrogen, Carlsbad, CA) to make 10% (weight/volume) tissue homogenates. Tissue homogenates were clarified by centrifugation and titrated in 24- and 96-well tissue culture plates containing MDCK cell monolayers, as described previously [21]. Titers are expressed as $\log_{10}$ TCID₅₀/g tissue.

2.6 Assessment of immunogenicity

HAI antibody titers in pre- and post-vaccination serum samples were determined by standard methods and were defined as the reciprocal of highest dilution of serum that completely inhibited the agglutination of erythrocytes by 4 HA units of virus antigen. The HAI titers of each group are presented as geometric mean titers (GMT). CA09 specific IgA titers in nasal wash of pre- and post-vaccination animals were determined by ELISA as described above. An optical density (OD) > 0.2 was considered to be positive.

2.7 Assessment of vaccine efficacy against CA09 wt challenge

To determine the efficacy of the CA09_{ca} vaccine, rhesus macaques were challenged with the CA09_{wt} virus and the level of replication of the challenge virus in the respiratory tract of vaccinated animals was compared with the titers in the mock-immunized group. Nasal washes and nasal swabs were collected on days 2 and 4 post-challenge. At necropsy on day 4, tracheal lavage, nasal turbinates, trachea, lungs and intestines were collected. Virus titers were determined as described above.

2.8 Flow cytometry

Single-cell suspensions from lung tissues were prepared as previously described [20, 22]. In brief, the lungs were digested for 15 min at 37°C in media containing 1 mg/ml collagenase (Roche Diagnostics GmbH, Mannheim, Germany) and 0.02 mg/ml DNase (Sigma-Aldrich, St. Louis, MO). The treated lungs were pressed through a cell strainer (BD Pharmingen, San Diego, CA) to obtain a single-cell suspension. The cells were stained with FITC-CD3, PE-CD4, APC-CD8, FITC-CD14, APC-CD11c, PE-CD11b (BD Pharmingen, San Diego, CA) and Biotinylated-CD68 (Biolegend, San Diego, CA). After fixing with 2% paraformaldehyde, the cells were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA) and FlowJo Software (Tree Star, Inc, Ashland, OR).

2.9 Statistics

The significance of differences between different groups was assessed using the unpaired Student's *t* test using Prism 5 (GraphPad Software, Inc. San Diego, CA); *p* values <0.05 were considered significantly different.

3. Results

3.1 Replication efficiency and pathogenicity of CA09wt and CA09ca viruses in African green monkeys (AGMs) and rhesus macaques

Groups of 12 AGMs and rhesus macaques were inoculated intranasally and intratracheally with 2×10^6 TCID₅₀ of the wild type pH1N1 virus (CA09_{wt}) or the live attenuated vaccine (CA09_{ca}). Four monkeys in each group were euthanized and organs were harvested on days 2, 4 and 7 post-infection (Fig. 1A). None of the AGMs and rhesus macaques that received these viruses developed clinical signs of infection such as increased body temperature, weight loss, fever or signs of respiratory distress. The CA09_{wt} virus replicated in the nasal

turbinates and trachea of experimentally inoculated AGMs and rhesus macaques. The CA09*wt* virus was detected in the nasal turbinates of all 4 animals on day 2 post-infection. The degree of replication of the CA09*wt* virus in the nasal turbinates and trachea was higher in rhesus macaques than in AGMs. Peak replication in the upper respiratory tract was observed on day 4 post-infection in rhesus macaques, and 2 days post-infection in AGMs (Fig. 1B and 1C). Replication of the CA09*ca* vaccine virus was severely restricted in the upper respiratory tract; virus was detected in the nasal turbinates of 1 of 4 AGMs and 4 of 4 rhesus macaques 2 days post-infection (Fig. 1B and 1C).

Evidence of replication of the CA09*wt* and CA09*ca* viruses was also observed in nasal swabs, nasal washes and tracheal lavages from monkeys on days 2, 4 and 7 post-infection. In AGMs, CA09*wt* virus was detected in nasal swabs but not from nasal washes and tracheal lavage samples. Virus was detected in nasal swabs of all 4 animals on day 4 post-infection (Fig. 2A). CA09*wt* virus was detected from nasal swabs, nasal washes and tracheal lavage samples of rhesus macaques; the mean virus titer in respiratory secretion samples from rhesus macaques was slightly higher on day 4 than on day 2 or day 7 post-infection (Fig. 2B). The CA09*ca* virus was not detected in respiratory secretions of AGMs or rhesus macaques.

The CA09*wt* virus replicated inconsistently in AGM lungs; viral replication was observed in only 4 AGMs. Replication was observed in all rhesus macaques that received the CA09*wt* virus and the peak of replication was seen on day 4 post-infection (Table 1). Replication of the CA09*ca* virus was not detected in the lungs of infected AGMs or rhesus macaques (data not shown) consistent with the temperature sensitive phenotype of the vaccine virus [23].

Interestingly, replication of CA09*wt* virus was observed by virus titration in the jejuno-ileal (1/12; 2.95 TCID₅₀/g) and ileocecal valve (2/12; 3.7 and 2.5 TCID₅₀/g) region of the small intestine of rhesus macaques on days 4 or 7 post-infection but not in AGMs.

3.2 Cellular composition in the lungs after infection with CA09*wt* and CA09*ca* viruses

To determine the cellular composition in lung tissues, single-cell suspensions were prepared and cell populations were detected using cellular makers. We determined the number of CD4⁺ T cells (CD4⁺CD3⁺), CD8⁺ T cells (CD8⁺CD3⁺) and macrophages (CD11b⁺CD11c⁺CD68⁺) in the lungs of CA09*wt* and CA09*ca* virus infected AGMs (Fig. 3A) and rhesus macaques (Fig. 3B) on days 2, 4 and 7 post-infection. The number of CD4⁺ and CD8⁺ T cells in the lungs of infected animals remained stable from days 2 to 7 post-infection. Interestingly, an increased number of macrophages were observed in the lungs of CA09*wt* virus infected AGMs (Fig. 3A left panel) and rhesus macaques (Fig. 3B left panel) but not in animals that received the CA09*ca* vaccine virus (Fig. 3A and 3B right panel).

3.3 Immunogenicity of CA09*ca* vaccine in rhesus macaques

We used rhesus macaques to study the immunogenicity and efficacy of the CA09*ca* vaccine because the CA09*wt* virus replicated more efficiently in this species (Fig. 1 and Table 1). Rhesus macaques were immunized with either 1 or 2 doses of vaccine (4 animals per group) (Fig. 4A). An additional 2 monkeys in each group were mock immunized with L-15. Four weeks post-vaccination, the animals that received 1 dose of the vaccine were challenged with the 2x10⁶ TCID₅₀/ml of CA09*wt* virus, which was previously shown to result in 100% infection in rhesus macaque (Table 1.), and the animals that received 2 doses of vaccine were challenged 4 weeks after the second dose of vaccine. pH1N1-specific IgA antibody was detected in nasal wash samples on days 28 and 56 post-immunization (Fig. 4B). Animals that received 2 doses of vaccine had higher pH1N1-specific IgA titers than animals that received one dose of vaccine. Serum samples were collected on days 0, 28 and 56 post-

immunization. On day 28, after the first dose of vaccine, all animals developed hemagglutination inhibition (HAI) antibodies in their sera, with titers ranging from 1:40 to 1:80 (Table 2). After the second dose of vaccine, the titer of HAI antibody was significantly higher (ranging from 160 to 320, Table 2). HAI antibodies were not detected in mock-immunized animals.

3.4 Efficacy of CA09ca vaccine in rhesus macaques

The protective efficacy of the CA09ca vaccine against homologous CA09 *wt* virus challenge was evaluated in respiratory tissues and secretions on day 4 after challenge. This time point was selected based on the replication kinetics of CA09 *wt* virus in rhesus macaques. Virus was detected in all tissues and secretions from the mock-immunized group (Table 2). In animals that received 1 dose of vaccine, challenge virus was detected in the nasal turbinates of one animal, while two other animals showed low titers of virus in the nasal swabs and high titers of virus in the lungs. Two doses of vaccine elicited better protective efficacy; virus was only detected in nasal swabs and lung tissue from one animal; this animal had a lower serum HI antibody titer than the other animals (HAI titer =160) (Table 2), and no pH1N1 specific IgA antibodies detected in the nasal wash (Figure 4B (day 56))

4. Discussion

In this study, we compared the pathogenesis of the 2009 pandemic H1N1 (CA09 *wt*) virus in two species of non-human primates, African green monkeys and rhesus macaques. Non-human primates support replication of influenza viruses for a short duration but virus replication may be detected without evidence of clinical illness. The more recent studies have been conducted in Old World monkeys; cynomolgus macaques and Rhesus macaques have been used to study HPAI H5N1 and 1918 pH1N1 virus infection. These monkeys exhibited systemic symptoms similar to those experienced by humans, including fever, lethargy, and the loss of appetite, but respiratory signs and symptoms were limited [14, 24, 25]. Cynomolgus macaques infected with a seasonal H3N2 virus did not show clinical signs of infection, despite the recovery of virus from nasal swabs and lung lavage [26] whereas seasonal H1N1 virus infected pigtailed macaques showed clinical signs of illness, including weight loss, nasal discharge and fever associated with lung pathology [15]. Thus, the demonstration of clinical illness in non-human primates is determined by the species, the route of inoculation and the influenza virus strain [27]. In our study, none of the animals receiving the pH1N1 virus developed clinical symptoms although viral replication was observed in the respiratory tract, which was similar to what was observed in ferrets and mice infected with the CA09 *wt* virus [4]. However, our findings differ from the observations previously reported in cynomolgus macaques. Cynomolgus macaques infected with the pH1N1 virus showed clinical signs of disease progression [11–13]. The discrepancy between our findings and those in cynomolgus macaques may be because the susceptibility of different species of non-human primates differs or because different routes and dose of inoculum were used. In two of the cynomolgus macaque studies, virus was administered by multiple routes including intratracheal, intranasal, ocular or conjunctival and oral simultaneously, whereas in our study we inoculated via I.T. and I.N. routes only. Heterogeneity of viral replication in different non-human primate species raises the possibility that host factors such as the influenza virus receptor pattern differs among non-human primate species. The distribution of influenza virus receptors in different non-human primate species needs to be explored further.

Lung consolidation has been described as a feature of severe influenza virus infection in humans [28–31]. Macrophages and neutrophils are prominent cell types associated with and that potentially mediate severe lung pathology following infection with highly virulent H5N1 and 1918 influenza viruses in mice [32]. Evidence of macrophage and neutrophil

infiltration in the lungs was also demonstrated in cynomolgus macaques [14, 24] infected with highly pathogenic H5N1 and 1918 viruses. In the present study, we determined the cellular composition in the lungs of AGMs and rhesus macaques following pandemic H1N1 virus infection. The cellular composition in the lungs of CA09 *wt* infected rhesus macaques (Fig. 3B) was similar to that observed in the lungs of mice infected with 1918 pH1N1 and highly pathogenic H5N1 viruses [32], where a dramatic increase of macrophages was observed in the lungs on day 7 post-infection. We did not observe macrophage infiltration in the lungs of CA09 *ca*-infected rhesus macaques. We suspect that this is because the CA09 *ca* virus is temperature sensitive and does not replicate in the lungs. Therefore, the infiltration of macrophages may be a consequence of the replication of wild type virus in lungs of AGMs and rhesus macaques. However, in the current study we did not determine the function of these cells in pH1N1 infection in AGMs and rhesus macaques.

We and others have demonstrated the efficacy and immunogenicity of the live attenuated CA09 *ca* vaccine in small animal models such as mice and ferrets [5, 33]. In this study, we examined the efficacy and immunogenicity of the CA09 *ca* vaccine in rhesus macaques because we found that these animals support CA09 *wt* virus replication better than AGMs. Because we observed no clinical signs of disease in this model, we evaluated the efficacy of vaccine based on protection from replication of the CA09 *wt* challenge virus. Although the CA09 *ca* vaccine showed minimal replication limited to the upper respiratory tract of rhesus macaques, serum HAI as well as H1N1-specific mucosal IgA antibodies were detected in animals that received only one dose of vaccine and the titers of these antibodies increased after the second dose. These results indicate that the CA09 *ca* vaccine is immunogenic in rhesus macaques. Animals receiving either one or two doses of vaccine were protected from CA09 *wt* virus challenge. We also observed an inverse correlation between serum HAI and nasal IgA antibodies and the degree of protection from replication of the challenge virus, suggesting that the humoral immune response plays a critical role in protection.

In summary, our results indicate that the virus as well as non-human primate species determines the pattern of influenza virus infection and that the live attenuated CA09 *ca* vaccine virus is immunogenic and efficacious in rhesus macaques.

Acknowledgments

This research was performed as part of a Cooperative Research and Development Agreement between the Laboratory of Infectious Diseases, NIAID and MedImmune. This work was supported in part by the Intramural Research Program of National Institute of Allergy and Infectious Diseases (NIAID), NIH. We would like to thank Jin Hong and George Kemble, MedImmune, Inc for critical reading of this manuscript.

We would like to thank Dr. Richard Herbert, Joanne Swerczek, Mark Szarowicz and staff of the NIH animal center at Poolesville for excellent technical support in the animal studies. We also thank Dr. Fernando Torres-Velez, Comparative Medicine Branch, NIH/NIAID/DIR and Dr. Ian Moore, LID/NIAID for technical assistance with necropsies.

References

1. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, et al. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science*. 2009; 325:197–201. [PubMed: 19465683]
2. Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van Kerkhove MD, Hollingsworth TD, et al. Pandemic potential of a strain of influenza A (H1N1): early findings. *Science*. 2009; 324:1557–61. [PubMed: 19433588]
3. Girard MP, Tam JS, Assossou OM, Kieny MP. The 2009 A (H1N1) influenza virus pandemic: A review. *Vaccine*. 2010; 28:4895–902. [PubMed: 20553769]

4. Min JY, Chen GL, Santos C, Lamirande EW, Matsuoka Y, Subbarao K. Classical swine H1N1 influenza viruses confer cross protection from swine-origin 2009 pandemic H1N1 influenza virus infection in mice and ferrets. *Virology*. 2010; 408:128–33. [PubMed: 20926110]
5. Chen GL, Min JY, Lamirande EW, Santos C, Jin H, Kemble G, et al. Comparison of a live attenuated 2009 H1N1 vaccine with seasonal influenza vaccines against 2009 pandemic H1N1 virus infection in mice and ferrets. *J Infect Dis*. 2011; 203:930–6. [PubMed: 21257740]
6. Suguitan AL Jr, Cheng X, Wang W, Wang S, Jin H, Lu S. Influenza H5 hemagglutinin DNA primes the antibody response elicited by the live attenuated influenza A/Vietnam/1203/2004 vaccine in ferrets. *PLoS One*. 2011; 6:e21942. [PubMed: 21760928]
7. Karron RA, Talaat K, Luke C, Callahan K, Thumar B, Dilorenzo S, et al. Evaluation of two live attenuated cold-adapted H5N1 influenza virus vaccines in healthy adults. *Vaccine*. 2009; 27:4953–60. [PubMed: 19540952]
8. Grizzard MB, London WT, Sly DL, Murphy BR, James WD, Parnell WP, et al. Experimental production of respiratory tract disease in cebus monkeys after intratracheal or intranasal infection with influenza A/Victoria/3/75 or influenza A/New Jersey/76 virus. *Infect Immun*. 1978; 21:201–5. [PubMed: 101457]
9. Murphy BR, Sly DL, Hosier NT, London WT, Chanock RM. Evaluation of three strains of influenza A virus in humans and in owl, cebus, and squirrel monkeys. *Infect Immun*. 1980; 28:688–91. [PubMed: 6772565]
10. Baskin CR, Garcia-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villan E, et al. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). *J Virol*. 2004; 78:10420–32. [PubMed: 15367608]
11. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, et al. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. *Nature*. 2009; 460:1021–5. [PubMed: 19672242]
12. Herfst S, van den Brand JM, Schrauwen EJ, de Wit E, Munster VJ, van Amerongen G, et al. Pandemic 2009 H1N1 influenza virus causes diffuse alveolar damage in cynomolgus macaques. *Vet Pathol*. 2010; 47:1040–7. [PubMed: 20647595]
13. Safronetz D, Rockx B, Feldmann F, Belisle SE, Palermo RE, Brining D, et al. Pandemic swine-origin H1N1 influenza A virus isolates show heterogeneous virulence in macaques. *J Virol*. 2011; 85:1214–23. [PubMed: 21084481]
14. Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD. A primate model to study the pathogenesis of influenza A (H5N1) virus infection. *Avian Dis*. 2003; 47:931–3. [PubMed: 14575089]
15. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, Sabourin PJ, Long JP, Garcia-Sastre A, et al. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. *Proc Natl Acad Sci U S A*. 2009; 106:3455–60. [PubMed: 19218453]
16. Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, et al. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. *Nature*. 2007; 445:319–23. [PubMed: 17230189]
17. Chen Z, Wang W, Zhou H, Suguitan AL Jr, Shambaugh C, Kim L, et al. Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. *J Virol*. 2010; 84:44–51. [PubMed: 19864389]
18. Stephenson I, Wood JM, Nicholson KG, Zambon MC. Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol*. 2003; 70:391–8. [PubMed: 12767002]
19. Chen GL, Lamirande EW, Jin H, Kemble G, Subbarao K. Safety, immunogenicity, and efficacy of a cold-adapted A/Ann Arbor/6/60 (H2N2) vaccine in mice and ferrets. *Virology*. 2010; 398:109–14. [PubMed: 20034647]
20. Chen GL, Lau YF, Lamirande EW, McCall AW, Subbarao K. Seasonal influenza infection and live vaccine prime for a response to the 2009 pandemic H1N1 vaccine. *Proc Natl Acad Sci U S A*. 2010; 108:1140–5. [PubMed: 21199945]

21. Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K. Evaluation of replication and pathogenicity of avian influenza A H7 subtype viruses in a mouse model. *J Virol.* 2007; 81:10558–66. [PubMed: 17634234]
22. Lau YF, Santos C, Torres-Velez FJ, Subbarao K. The magnitude of local immunity in the lungs of mice induced by live attenuated influenza vaccines is determined by local viral replication and induction of cytokines. *J Virol.* 2010; 85:76–85. [PubMed: 20962087]
23. Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, et al. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. *Virology.* 2003; 306:18–24. [PubMed: 12620793]
24. Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (*Macaca fascicularis*). *Vet Pathol.* 2003; 40:304–10. [PubMed: 12724572]
25. Chen Y, Deng W, Jia C, Dai X, Zhu H, Kong Q, et al. Pathological lesions and viral localization of influenza A (H5N1) virus in experimentally infected Chinese rhesus macaques: implications for pathogenesis and viral transmission. *Arch Virol.* 2009; 154:227–33. [PubMed: 19130169]
26. Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lovgren-Bengtsson K, Claas EC, et al. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. *J Gen Virol.* 1997; 78 (Pt 4):757–65. [PubMed: 9129647]
27. O'Donnell CD, Subbarao K. The contribution of animal models to the understanding of the host range and virulence of influenza A viruses. *Microbes Infect.* 2011; 13:502–15. [PubMed: 21276869]
28. Gill JR, Sheng ZM, Ely SF, Guinee DG, Beasley MB, Suh J, et al. Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections. *Arch Pathol Lab Med.* 2010; 134:235–43. [PubMed: 20121613]
29. Yuen KY, Chan PK, Peiris M, Tsang DN, Que TL, Shortridge KF, et al. Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. *Lancet.* 1998; 351:467–71. [PubMed: 9482437]
30. Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J, et al. H5N1 infection of the respiratory tract and beyond: a molecular pathology study. *Lancet.* 2007; 370:1137–45. [PubMed: 17905166]
31. Carmona F, Carlotti AP, Ramalho LN, Costa RS, Ramalho FS. Evidence of Renal Infection in Fatal Cases of 2009 Pandemic Influenza A (H1N1). *Am J Clin Pathol.* 2011; 136:416–23. [PubMed: 21846917]
32. Perrone LA, Plowden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS Pathog.* 2008; 4:e1000115. [PubMed: 18670648]
33. Yang P, Duan Y, Wang C, Xing L, Gao X, Tang C, et al. Immunogenicity and protective efficacy of a live attenuated vaccine against the 2009 pandemic A H1N1 in mice and ferrets. *Vaccine.* 2011; 29:698–705. [PubMed: 21111782]

Highlights

- The 2009 pH1N1 virus replicates to higher titer in rhesus macaques than AGMs
- The live attenuated pH1N1 influenza virus vaccine is immunogenic in rhesus macaques
- The pH1N1 LAIV protects rhesus macaques from wild-type virus challenge

Figure 1.

Replication of CA09 *wt* and CA09 *ca* viruses in the upper respiratory tract of non-human primates. Schematic for replication study (A). The titers (expressed as TCID₅₀/g of tissue) of CA09 *wt* (●) and CA09 *ca* (○, ◇) virus in the upper respiratory tract (nasal turbinates and trachea) of AGMs (B) and rhesus macaques (C). Virus titer in nasal turbinates and trachea in 4 monkeys per group euthanized on day 2, 4 and 7 post-infection. The mean titer from each group is represented as a solid line. The dashed line represents the lower limit of detection.

A.

B.

Figure 2.

Viral replication in respiratory secretions. Replication of CA09 wt (●, ◆) and CA09 ca (○, ◇) virus in nasal swabs, nasal washes and tracheal lavage samples of AGMs (A) and rhesus macaques (B). Viral titers in secretions are expressed as log₁₀TCID₅₀/mL. The mean titer from each group is represented as a solid line. The dashed line represents the lower limit of detection.

A.

B.

Figure 3.

Cellular composition in lung homogenates of AGMs (A) and rhesus macaques (B) infected with CA09 *wt* and CA09 *ca* viruses. Monkeys were infected with CA09 *wt* or CA09 *ca* and euthanized on days 2, 4 or 7 post-infection. A single-cell suspension of the lung tissue was prepared and the cells were stained with cellular markers (CD3, CD8, CD4, CD11b, CD11c and CD68). The bars and error bars represent the mean and standard errors for each group.

*, $p < 0.05$; Student's *t* test.

Figure 4.

Schematic for the vaccine immunogenicity and efficacy study (A). Mucosal IgA after immunization, anti-H1N1 IgA antibody in nasal wash of rhesus macaques was detected on days 0, 28 and 56 post-vaccination by ELISA assay. The IgA titer is shown as optical density at 450 nm (B). The dashed line at an O.D of 0.2 represents the cut-off value for this ELISA.

Table 1

Replication of H1N1 CA09 *wt* virus in lung tissues.

Portion of lung sampled	Viral replication frequency (titer in positive samples) detected on indicated day post infection			
	African Green Monkeys		Rhesus Macaques	
	Day 2	Day 4	Day 7	
Cranial right distal	- ^c	-	-	2/4 (2.0,2.0)
Cranial right proximal	-	1/4 ^a (3.5) ^b	-	3/4 (4.2,3.7,2.5)
Caudal right distal	-	-	-	1/4 (4.2)
Caudal right proximal	-	1/4 (2.5)	1/4 (2.95)	4/4 (3.7,2.5,4.5,3.9)
Cranial left distal	-	-	-	2/4 (2.5,2.0)
Cranial left proximal	-	-	-	3/4 (4.2,3.7,2.5)
Caudal left distal	1/4 (4.7)	-	-	3/4 (3.2,2.5,4.2)
Caudal left proximal	-	-	-	2/4 (2.0,2.5)
				1/4 (2.0)
				2/4 (3.2,2.5)
				-
				2/4 (2.95,2.0)
				2/4 (2.0,2.0)

^aNumber of animals in which virus was found in the lungs/total number (4) of animals in each group.

^bVirus titer in the lungs expressed as log₁₀TCID₅₀/g.

^cVirus was not detected in any of the 4 animals; the lower limit of detection was 10^{1.5} TCID₅₀/g.

Table 2

Immunogenicity and efficacy of the CA09ca vaccine in rhesus macaques

Immunogen	Viral replication frequency (titer in positive samples) in indicated tissue							Geometric mean titer (range)
	Secretions (log ₁₀ TCID ₅₀ /ml)							
	NT	Trachea	Lung	Intestine	Nasal wash	Nasal swab	Tracheal lavage	Day 0
L-15 (Diluent control)	4/4 ^c (5.7,5.9, 5.2,5.7)	4/4 (4.9,4.2, 3.9,4.4)	4/4 (4.9,5.7, 4.5,4.9) ^f	2/4 (4.2,2.5)	2/4 (1.0,1.5)	4/4 (1.7,1.7, 1.5,1.7)	1/4 (1.5)	<10
1 dose ^a CA09 <i>ca</i>	1/4 (4.2)	0/4	2/4 (4.9,5.2)	0/4	0/4	2/4 (1.0,1.0)	0/4	<10
2 doses ^b CA09 <i>ca</i>	0/4	0/4	1/4 (4.5)	0/4	0/4	1/4 (1.0)	0/4	<10

^aRhesus macaques were immunized with a single dose of CA09ca vaccine and were challenged with the CA09wt virus 4 weeks post-vaccination.

^bRhesus macaques were immunized with 2 doses of CA09ca vaccine. The second dose of vaccine was given 4 weeks after the first dose and animals were challenged with CA09wt 4 weeks after the second immunization.

^cNumber of animals in which virus was detected out of total animals (n=4) in each group.

^dViral titer in tissue or respiratory secretion samples determined by titration on MDCK cells. The lower limit of detection was 10^{1.5} TCID₅₀/g for tissue samples and 10^{0.5} TCID₅₀/ml for secretions.

^eFour weeks after dose 1 or 2, serum samples were collected for determining HAI antibodies against the CA09wt virus.

^fAverage virus titers from the influenza-positive lung sections.