Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production

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Accepted 19 August 2009. Published Online 9 October 2009.

Background Highly pathogenic H5N1 avian influenza viruses currently circulating in birds have caused hundreds of human infections, and pose a significant pandemic threat. Vaccines are a major component of the public health preparedness for this likely event. The rapid evolution of H5N1 viruses has resulted in the emergence of multiple clades with distinct antigenic characteristics that require clade-specific vaccines. A variant H5N1 virus termed clade 2.3.4 emerged in 2005 and has caused multiple fatal infections. Vaccine candidates that match the antigenic properties of variant viruses are necessary because inactivated influenza vaccines elicit strain-specific protection.

Objective To address the need for a suitable seed for manufacturing a clade 2.3.4 vaccine, we developed a new H5N1 pre-pandemic candidate vaccine by reverse genetics and evaluated its safety and replication *in vitro* and *in vivo*.

Methods A reassortant virus termed, Anhui/PR8, was produced by reverse genetics in compliance with WHO pandemic vaccine development guidelines and contains six genes from A/Puerto Rico/8/34 as well as the neuraminidase and hemagglutinin (HA) genomic segments from the A/Anhui/01/2005 virus. The multi-basic cleavage site of HA was removed to reduce virulence.

Results The reassortant Anhui/PR8 grows well in eggs and is avirulent to chicken and ferrets but retains the antigenicity of the parental A/Anhui/01/2005 virus.

Conclusion These results indicate that the Anhui/PR8 reassortant lost a major virulent determinant and it is suitable for its use in vaccine manufacturing and as a reference vaccine virus against the H5N1 clade 2.3.4 viruses circulating in eastern China, Vietnam, Thailand, and Laos.

Keywords Pandemic influenza, H5N1 vaccine, seed virus.

Please cite this paper as: Dong et al. (2009) Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production. *Influenza and Other Respiratory Viruses* 3(6), 287–295.

Introduction

Influenza viruses cause seasonal epidemics of acute respiratory disease with most severe consequences in pediatric and geriatric populations. Sporadically, influenza A viruses with a novel hemagglutinin (HA) subtype cause pandemics with serious public health consequences. Pandemics are caused by influenza viruses containing surface protein(s) for which the human population has little or no immunity, and endowed with the ability to spread in the human population. The three influenza pandemics of the 20th century involved the transmission of influenza viruses from animals to humans.¹ The most severe pandemic of the previous century was the 'Spanish Influenza' of 1918–1919, which spread globally in just 1 year and caused at least 40 million deaths.¹

The pandemic of 1957 was caused by an H2N2 reassortant virus containing avian HA, neuraminidase (NA), and polymerase basic protein 1(PB1) genes in the background of genes belonging to a circulating human H1N1 virus and rapidly displaced the circulating H1N1 viruses.^{2–4} In 1968, an H3N2 reassortant virus containing avian HA and PB1 genes with the remaining genes from the contemporary H2N2 virus emerged to cause a pandemic.^{2,4,5} Another influenza pandemic could occur at any time and effective public health interventions based on appropriate preparedness are needed to diminish morbidity and mortality.

Numerous different subtypes of the influenza A virus exist in nature, resulting from combinations of 16 serotypes of HA (H1–H16) and nine serotypes of NA (N1–N9).⁶ The human population has immunity to only a few of the

known serotypes. To date, viruses with HA subtypes (H1, H2, H3, H5, H6, H7, and H9) and four NAs (N1, N2, N3, and N7) have been isolated (or antibodies against them have been detected) from humans.^{4,7} Among these, subtype H5, H6, H7, and H9 viruses of avian origin have infected humans and are considered potential pandemic threats.

Highly pathogenic avian influenza (HPAI) H5N1 viruses currently circulating in birds have resulted in more than 400 instances of H5N1 transmission to humans since 2004, suggesting that this virus could initiate the next pandemic.⁸ The HA sequences of avian H5N1 viruses have diverged into ten distinct phylogenetic clades (genetic groups).⁹ Genetic divergence into clades is also correlated with antigenic drift and often antibodies generated against one clade do not efficiently inhibit hemagglutination or neutralize a virus from another clade.^{10,11} As an additional complication, some clades have evolved further into second and third order clades (subclades). Most currently circulating H5N1 strains that have infected humans belong to four serologically distinct antigenic groups (clades 1, 2.1, 2.2, and 2.3.4).⁹

The impact of viral evolution on the antigenic structure of HA becomes evident upon comparing the HA amino acid sequences of the major H5N1 clades. A large proportion of the amino acids that constitute putative antigenic sites in the HA are polymorphic suggesting that the antigenicity of the four major clades has drifted.^{12,13} The consequence of such structural evolution is reflected in the reactivity of these viruses with panels of ferret antisera which are used to assess antigenic differences which may trigger the need to prepare a more closely matched vaccine candidate virus.^{10,14}

In this study, we analyzed the genetic and antigenic properties of a clade 2.3.4, subtype H5N1 virus which was then utilized for the derivation of a vaccine candidate by reassortment with the A/Puerto Rico/8/1934 (PR8) strain, which provided the polymerase, NP, M, and NS genes. This virus was expected to be antigenically representative of the entire clade 2.3.4 and to provide some cross-reactive immunity outside this clade. In addition, since it has the internal genes from PR8 which should confer high growth in chicken embryo and partial attenuation in humans, it was expected to grow to reasonably high viral titers and to lack virulence.¹⁵ These properties were evaluated to determine the suitability of this reassortant to develop a seed virus for the preparation of an inactivated subunit or split vaccine, the only type of H5N1 vaccine currently approved by the FDA, for use in the event of a pandemic.

Materials and methods

Cells and viruses

Madin–Darby canine kidney and 293T human embryonic kidney cells were obtained from the American Type Cul-

ture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). Vero cells from a working cell bank qualified for vaccine production were described previously.¹⁶ Vero cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% irradiated FBS (Cambrex, East Rutherford, NJ, USA) and 50 µg/ml Neomycin (Sigma, St. Louis, MO, USA). Primary chicken embryo fibroblast (CEF) and primary chicken kidney (PCK) cells were obtained from Charles River Laboratories, Wilmington, MA, USA. Influenza virus A/Anhui/1/2005 (H5N1) is a HPAI virus isolated from a fatal case of a pregnant woman in the Anhui province, China during 2005.¹⁷ The virus was isolated from a tracheal aspirate, propagated in 10-day-old embryonated chicken eggs and its viral RNA extracted in a biosafety level (BSL)-3-enhanced laboratory. Harvested virus was quantified with a standard HA assay using a 0.5% turkey erythrocyte cell suspension¹⁸ and also in eggs and CEF cells.

Sequence analysis of HA and NA genes, PCR amplification, mutagenesis, and cloning

Influenza viral genomes were sequenced as previously described.¹⁹ Briefly, RNA was extracted with the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA), and used for RT-PCR (OneStep RT/PCR Kit; Qiagen). The sequencing reaction was performed using BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Automated sequencing was performed using an ABI 3100 Genetic Analyzer. To clone the HA and at the same time remove the multi-basic cleavage site, overlapping PCR was performed using two sets of primers; one set corresponding to HA1 (5'-GATCGCTCTTCAGGGAGCAAAAGCAGGGG-3' and 5'-CTAGGCTCTTCTTCGTTCTCGTAGAGGACTATTTCTGAG-3') and other corresponding to HA2 (5'-AGTCGCTCTTCGCGAGGACTATTTGGAGCTATAGCAGG-3' and 5'-ACTGGCTCTTCTATTAGTAGAAACAAGGGTGTTTTTAACTAC-3'). For the amplification of the NA gene, we used the following universal primers which were modified from Hoffmann *et al.*²⁰ 5'-GATCGCTCTTCAGGGAGCAAAAGCAGGAGT-3' and 5'-ACTGGCTCTTCTATTAGTAGAAACAAGGAGTTTTT-3'. The amplified PCR products for HA and NA genes were digested with restriction enzyme *SapI* and ligated into the bidirectional transcription plasmid pCIPolISapIT (modified from pPolISapIT).¹⁹

Plasmid DNA

The plasmids containing the PR8 internal genes were modified from the pPolISapIT-derived PR8 plasmids described previously²¹ by transferring regulatory elements including the human polymerase I promoter, mouse terminator, and PR8 genome sequences into the pCDNA3.1⁺ mammalian expression vector (Invitrogen).

Virus recovery by DNA transfection

Plasmids containing HA and NA genes of A/Anhui/1/2005 (H5N1) together with plasmids carrying the six remaining genes, PB2, PB1, PA, NP, M, NS, of PR8 virus origin were used to transfect certified Vero cells using TransIT (Mirus, Madison, WI). Twenty four hours after transfection, PCK cells (Charles River Laboratories) were added to the Vero cells to support amplification of any released virus. The transfected cells and supernatants were harvested 48 hours later and 0.2 ml inoculated into the allantoic cavity of 10-day-old embryonated eggs. Eggs were incubated at 35°C for 2 days and allantoic fluid was analyzed by HA assay for the presence of virus. After one additional egg passage (E2), the infectivity and virion content was determined by titration in chicken embryos and HA assays, respectively. The former was expressed as EID₅₀ and was calculated using the Reed–Muench method from endpoint dilution.²² The HA titer was determined as described previously using turkey erythrocytes.²³ All experiments described in this manuscript were performed with a virus stock derived in Vero cells and passaged twice in eggs (V1E2).

Plaque formation on chicken embryo fibroblast cells with or without trypsin

The viral plaque characteristics and titer were determined on CEF (Charles River Laboratories) cells as described previously.²⁴ CEF cells were grown on six-well culture plates in DMEM supplemented with 10% FBS and antibiotics (100 µg/ml penicillin and 100 µg/ml streptomycin). After inoculation with serial dilutions of the virus stock, cell monolayers were overlaid with 0.8% agarose in DMEM without serum, with or without 0.5 µg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Plates were incubated for 48 hours and cells were stained with 0.1% crystal violet to visualize plaques.

Chicken embryo lethality test

Ten-day-old chicken embryos were inoculated in the allantoic sac with 0.1 ml of log₁₀ dilutions of each virus preparation with known infectious titers. The embryo viability was recorded at 48 hours post-inoculation. The virus dose that caused death in 50% of embryos was calculated by the method of Reed and Muench²² and reported as the Median Chicken Embryo Lethal Dose (CELD₅₀).

Pathogenicity for chickens

Groups of eight 4-week-old specific pathogen-free Plymouth White Rock chickens were inoculated with the reassortant virus, Anhui/PR8, or wild type A/Anhui/01/2005 virus, at a standard dose (10^{8.8} EID₅₀; 0.2 ml of a 1:10 dilution of stock virus) by the intravenous (i.v.) route and observed for 14 days.²⁵ Groups of eight chickens were also

inoculated intranasally (i.n.) with 10⁶ EID₅₀ in 0.1 ml of each virus and observed for 14 days.

Ferret inoculation studies

Six male ferrets (8-month old) that were serologically negative for currently circulating influenza A viruses were used to evaluate the outcome of infection with Anhui/PR8 virus or the reverse genetics derived A/Puerto Rico/8/34 (PR8-RG). Each ferret was i.n. inoculated with 10⁶ or 10^{6.3} EID₅₀ of Anhui/PR8 virus or the PR8-RG virus, respectively. Three ferrets were killed at 3 days post-inoculation (d.p.i.) and their spleen, lungs, whole blood, nasal turbinates, and brain were harvested for virus titration. The remaining three ferrets were monitored for clinical signs for 14 days and nasal washes were collected at 1, 3, 5, and 7 d.p.i. for virus titration. Infectious virus titers were determined by inoculation of embryonated eggs.

Antigenic characterization by HI assay

Antigenic characterization of relevant H5N1 viruses was performed using reference ferret antisera in a hemagglutination inhibition (HI) assay with turkey erythrocytes as previously described.²⁶

Results

Emergence and antigenic characteristics of clade 2.3.4 H5N1 viruses in Asia

The rapid evolution of HPAI H5N1 viruses circulating in birds has led to the emergence of multiple clades.⁹ To identify an appropriate virus representative of clade 2.3.4 for vaccine development, a group of related clade 2 viruses were subjected to phylogenetic and antigenic analysis. A/Anhui/01/2005 virus had close phylogenetic relationship to other viruses within clade 2.3.4 (Figure 1), suggesting that it is a suitable representative of the HA genetic diversity within in this clade.

Post-infection reference ferret antisera was generated against A/Anhui/01/2005 virus and was used in HI assays to determine the antigenic relatedness of A/Anhui/01/2005 virus compared with other viruses in the clade and *vice versa*. Antisera to A/Anhui/01/2005 showed strong inhibition of hemagglutination by the homologous antigen, as well as A/Indonesia/5/2005 virus (clade 2.1), but was minimally inhibitory for viruses of clade 1 and 2.2, as well as some 2.1 viruses (Table 1). Most importantly, A/Anhui/01/2005 virus antisera yielded high HI titers with all the clade 2.3.4 viruses tested, indicating that this strain served as a representative antigen for the generation of a pre-pandemic vaccine candidate reassortant virus.

Reassortant virus rescue and growth in embryonated eggs

To generate a PR8 reassortant virus displaying the A/Anhui/01/2005 virus surface proteins, we amplified both the

Figure 1. Phylogenetic relationships of the A/Anhui/1/2005 hemagglutinin gene to other recent Eurasian H5N1 viruses. The phylogeny was generated by neighbor-joining analysis using the Kimura 2-parameter in MEGA 4 (<http://www.megasoftware.net/mega.html>). Bootstrap values at each node represent 1000 replicates. Scale bar represents a nucleotide distance of 0.005. Viruses included in the antigenic characterization (Table 1) are underlined; A/Anhui/1/2005 and the clade designation are highlighted in yellow.

HA and NA genes and cloned them into a reverse genetics plasmid. Four basic amino acid residues were removed from the HA cleavage site by directed mutagenesis. After plasmid transfection, virus recovered from Vero cell cul-

tures, termed Anhui/PR8 was then passaged twice in embryonated chicken eggs.

To determine whether the rescued reassortant Anhui/PR8 virus retained the amino acid sequence from

Table 1. Hemagglutination inhibition assay of H5N1 viruses

Reference antigens	Reference ferret antisera											
	Clade designation											
	1	1	2.1	2.2	2.2	2.2	2.2	2.3.4	2.3.4	2.3.4	2.3.4	2.3.4
	bg/QI ANH/1 VN/1203 TH/16 IND/5 ws/MG tk/TK N-PR8 EG/321 ANH/1 -PR8 jwy/HK dk/LO ck/VN											
A/Vietnam/1203/2004	320	40	<10	<10	10	20	<10	<10	<10	<10	20	160
A/Thailand/16/2004	40	160	<10	<10	10	20	<10	<10	<10	<10	<10	160
A/Indonesia/5/2005-PR8	<10	<10	640	160	10	40	40	80	20	<10	<10	20
A/whooper swan/Mongolia/244/2005	<10	<10	320	640	80	320	160	20	40	80	10	10
A/turkey/Turkey/1/2005 PR8	<10	40	320	1280	320	320	160	40	80	80	40	20
A/barheadedgoose/Qinghai/1A/2005-PR8	<10	<10	320	640	160	640	320	20	80	40	20	20
A/Egypt/321/2007	<10	<10	160	320	80	160	640	10	40	40	20	20
A/Anhui/1/2005	20	<10	160	<10	20	40	10	1280	320	160	320	320
A/Anhui/1/2005-PR8	80	10	160	<10	40	80	10	2560	320	160	640	160
A/Japanese white eye/Hong Kong/1 03 8/2006	<10	<10	160	<10	20	160	160	640	320	640	320	80
A/duck/Laos/3295/2006	<10	<10	<10	<10	<10	20	10	320	80	80	80	80
A/chicken/Vietnam/NCVD-35/2008	<10	<10	<10	<10	<10	10	<10	80	40	40	80	320

Abbreviations: VN/1203, A/Vietnam/1203/2004; TH/16, A/Thailand/16/2004; IND/5, A/Indonesia/5/2005; ws/MG, A/whooper swan/Mongolia/244/2005; tk/TK, A/turkey/Turkey/1/2005; bg/QIN-PR8, A/barheaded goose/Qinghai/1A/2005-(H5N1)-PR8; EG/321, A/Egypt/321/2007; ANH/1, A/Anhui/1/2005; ANH/1-PR8, A/Anhui/1/2005-(H5N1)-PR8; jwy/HK, A/japanese white eye/Hong Kong/1038/2006; dk/LO, A/duck/Laos/3295/2006; ck/VN, A/chicken/Vietnam/NCVD-35/2008.

Table 2. Deletion of the virulence-inducing cleavage motif in HA of A/Anhui/01/2005

Amino acid sequence at cleavage site	
	↓
A/Anhui/01/2005	G L R N S P L R E R R K R G L F
Anhui/PR8	G L R N S P L R E R - - - G L F
A/CK/Italy/22A/1998	G P R N V P Q K E T R - - - G L F

↓Denotes proteolytic cleavage site.

the parental virus surface glycoproteins, viral nucleic acid from the second egg passage was sequenced. The HA and NA proteins of the Anhui/PR8 virus were identical to that of A/Anhui/01/2005 virus except for the modified HA cleavage site which displayed the desired four amino acid deletion (R-R-K-R) in the multibasic cleavage motif (Table 2). The newly created cleavage motif resembles those found in low pathogenic H5N1 influenza viruses (e.g., A/chicken/Italy/22A/98; Table 2) and complies with the WHO guidance for the development of pre-pandemic candidate vaccine viruses.¹⁰

To determine the ability of the second egg passage Anhui/PR8 reassortant virus to replicate to acceptable yields for vaccine production, its yield in embryonated eggs was determined by different methods. As shown in Table 3,

Table 3. Hemagglutination and infectivity titers of reassortant viruses

Virus	Passage history*	HA titer [†]	Infectivity titer	
			Eggs [‡]	MDCK [‡]
Anhui/PR8	V1E2	1:640	10 ^{8.5} EID ₅₀ /ml	10 ⁹ PFU/ml
Indo5/PR8	V1E2	1:1024	10 ^{9.5} EID ₅₀ /ml	10 ⁸ PFU/ml

*V1E2: produced by transfection in Vero cells and two passages in eggs.

[†]With turkey erythrocytes.

[‡]Host system used for titration.

MDCK, Madin-Darby canine kidney.

Anhui/PR8 virus demonstrated a high yield of viral infectivity (EID₅₀ and plaque forming unit (PFU)) and antigen (HA) from propagation in eggs. These results suggest that the reassortant virus replicated to levels that are likely to be scalable for industrial manufacturing of an influenza vaccine in embryonated eggs. The A/Indonesia/5/2005-(H5N1)-PR8 (Indo5/PR8) reassortant virus, which was successfully manufactured in eggs as split vaccine (Table 3) displayed similar replication characteristics as the Anhui/PR8 virus (M. Perdue, DHHS, personal communication).

Table 4. Trypsin-dependent plaque formation on CEF cells

Virus	Plaque formation on CEF cells			
	With trypsin		Without trypsin	
	PFU/ml	Diameter (mm)	PFU/ml	Diameter (mm)
Anhui/PR8	10 ^{8.3}	3	≤1	NA
A/Anhui/01/2005	10 ^{8.54}	2.5–3	10 ^{8.6}	2–2.5

NA, not applicable.

Trypsin-dependent plaque formation on CEF cells

Highly pathogenic viruses can undergo multicycle *in vitro* replication in the absence of trypsin due to the presence of a multibasic cleavage motif in HA that is readily cleaved by furin-like proteases expressed by all vertebrate cells.^{1,27} The genetic changes introduced into the newly developed pre-pandemic candidate vaccine viruses are expected to impart dependence on trypsin for growth in chicken fibroblasts, which is a property displayed by low pathogenicity viruses. Indeed, Anhui/PR8 virus failed to form plaques on CEF cell cultures lacking trypsin (Table 4). In the presence of trypsin, the reassortant virus was capable of forming plaques on CEF cells suggesting that the absence of plaques in cultures devoid of trypsin was not due to a defect inherent to the reverse genetics derivation (Table 4). These results indicate that Anhui/PR8 virus lacks an essential virulence determinant of HPAI as evidenced by a strict trypsin dependence for plaque formation on CEF cells.^{1,27}

Chicken embryo lethality test

Highly pathogenic avian influenza viruses are typically lethal to chicken embryos and death can be observed as early as 24 hours post-inoculation. To determine the lack of pathogenicity of Anhui/PR8 virus as compared to its wild type counterpart, 10-day-old chicken embryos were inoculated with reassortant or wild type viruses. High proportions of viable embryos were noted in groups at 24 hours after inoculation with relatively high doses of Anhui/PR8 virus and at 48 hours the CELD₅₀ for the reassortant virus was ≥10^{5.3} PFU/0.1 ml (data not shown and Table 5). In contrast, wild type A/Anhui/01/2005 virus was highly lethal to embryos at very low viral doses of virus; the CELD₅₀ was ≤10^{-0.39} PFU/0.1 ml at 48 hours after inoculation (Table 5), in agreement with previous reports.¹⁵

Pathogenicity testing in chickens

To determine the pathogenic potential of the reassortant virus in birds, groups of chickens were inoculated by either the i.v. or i.n. routes with 10^{8.8} or 10⁶ EID₅₀, respectively,

Table 5. Virulence assessment in chicken embryos and chickens

Virus	CELD ₅₀ *	Route	Dose†	IVPI	Morbidity	Mortality (MDT)
Anhui/PR8	≥10 ^{5.3}	IV	10 ^{8.8}	0.0	0/8	0/8
A/Anhui/01/2005	≤10 ^{-0.39}	IV	10 ^{8.8}	3.0	8/8	8/8 (1 d)

*CELD₅₀: 50% chicken embryo lethal dose; PFU/ml.

†Dose administered expressed in EID₅₀ (50% egg infectious dose) per 0.1 ml.

IV, intravenous; IVPI, intravenous pathogenic index; MDT, mean death time; d, days.

of Anhui/PR8 virus or its wild type parent. All chickens inoculated with the wild type parental virus, A/Anhui/01/2005, died with a mean death time of 1 day after inoculation by the i.v. route and 2.1 days by the i.n. route (Table 5). Mortality was 100% in the groups inoculated with wild type virus via either route. Postmortem examinations revealed lesions typical of HPAI infection, including petechial hemorrhages on viscera, hemorrhage of comb and wattle, and severe lung congestion and hemorrhage with interstitial pneumonia (data not shown) as reported previously.²⁸ In contrast, all chickens inoculated with Anhui/PR8 virus remained healthy throughout the 14-day observation period. Virus was isolated from both oral and cloacal swabs from birds inoculated with the wild type virus, but not from animals inoculated with the reassortant (data not shown). A/Anhui/01/2005 virus was also isolated from brain, lung, heart and kidney tissues of the inoculated chickens. In contrast, no virus was detected in either oral or cloacal swabs or from any of the above tissues in chickens inoculated with Anhui/PR8 virus (data not shown). Our results demonstrate the lack of pathogenicity of the H5N1 reassortant pre-pandemic candidate vaccine virus in chickens, indicating that this virus does not pose a major threat to domestic or wild birds.

Safety testing in ferrets

Ferrets infected with HPAI H5N1 viruses may display signs of lethargy, severe weight loss, and respiratory or neurological symptoms.^{29,30} To determine the pathogenic potential of the reassortant virus, six 8-month-old ferrets were inoculated with Anhui/PR8 or PR8-RG virus. None of the ferrets infected showed severe signs of disease over the 14 d.p.i. observation period, although they did show a 1–3% weight loss. A peak mean viral titer of 10^{3.5} EID₅₀/ml was detected in nasal washes collected from ferrets infected with Anhui/PR8 virus at 5 d.p.i. but virus was cleared from nasal washes by 7 d.p.i. in two of three ferrets. Viral titers

Table 6. Detection of infectious virus in ferret tissues at 3 days post-inoculation

Virus	Nasal turbinates	Lungs	Spleen	Brain
Anhui/PR8	1/3*	0/3	0/3	0/3
PR8-RG	3/3	1/3	0/3	0/3

*Number of animals with infectious virus/number tested.

ranged from $10^{2.3}$ to $10^{5.5}$ EID₅₀/ml in nasal washes from PR8-RG virus-infected ferrets on 1, 3, and 5 d.p.i. and one ferret at 7 d.p.i. (data not shown). Virus isolation studies from inoculated animals are presented in Table 6. Anhui/PR8 virus was detected at day 3 p.i. in the nasal turbinates and in the olfactory bulb of one of three ferrets but virus was not detected in the lungs, spleen, brain (posterior to the olfactory bulb) or whole blood of any of the inoculated ferrets, demonstrating that the reassortant vaccine candidate virus is not pathogenic to ferrets, a critical safety parameter for H5N1 vaccines.

Antigenic analysis of reassortant virus

To determine if the Anhui/PR8 reassortant virus had retained the antigenic characteristics of the A/Anhui/01/2005 donor virus we performed reciprocal HI assays using ferret antisera to each of these viruses (Table 1). For both wild type and reassortant viruses, homologous sera resulted in the same relative titers of hemagglutination inhibition. But more importantly, the convalescent or post-infection ferret serum to the A/Anhui/01/2005 virus inhibited hemagglutination by Anhui/PR8 virus to a similar titer as that against the immunizing wild type virus (Table 1). Likewise, the antisera to Anhui/PR8 virus inhibited hemagglutination by A/Anhui/01/2005 virus to a similar extent as the immunizing virus. A significant difference in antigenicity is typically defined as HI titer difference of at least fourfold. Therefore, these results indicate that within the limits of sensitivity of the HI assay, Anhui/PR8 virus maintained the antigenic characteristics of the parental virus.

Discussion

In contrast to the predominance of only a few major clades of each subtype of seasonal human influenza viruses,^{31,32} multiple clades of H5N1 co-circulate in birds in Asia, Africa, and Europe. Evolutionary studies of H5N1 virus suggest that although some lineages have become extinct, there are multiple lineages still co-circulating that can be grouped in diverse clades.⁹ Phylogenetic analysis is crucial

to understand how H5N1 evolves and phylogenetic divergence often goes hand in hand with changes in antigenicity which determine vaccine strain selection. Inactivated influenza vaccines are thought to elicit strain-specific protection.^{33,34} Therefore, co-circulation of multiple antigenically distinct H5N1 virus clades presents a formidable challenge to pandemic preparedness efforts involving vaccine stockpiles. Any of the viruses in the various H5N1 virus clades could potentially initiate a pandemic.¹⁰ By matching the pre-pandemic vaccine candidate viruses to major circulating H5N1 virus antigenic groups, an updated library of vaccine reference viruses is readily available for immediate use. Such a repository enables rapid development of seed viruses for manufacturing of pre-pandemic vaccine for stockpiles or for expedited production of vaccine^{10,14} and immunization in case of pandemic emergency.

H5 viruses of four antigenically distinct clades have been isolated from humans since 2004. Clades 1, 2.2, 2.1.3, and 2.3.4 have been isolated from clinical cases and public health efforts have concentrated in the derivation of reassortant viruses belonging to these clades.¹⁰ The first candidate H5N1 reassortant viruses were derived from clades 1 and 2.1 viruses. Later, the isolation of viruses belonging to clades 2.2 and 2.3.4 in humans motivated the generation of additional reassortant viruses. The A/Anhui/01/2005 virus belonging to clade 2.3.4 and isolated from a pregnant woman in the Anhui province of China, was chosen as a representative reference strain to make the Anhui/PR8 reassortant virus.¹⁷ Clade 2.3.4 viruses are predominant in China (and also Vietnam, Thailand, and Laos) and have resulted in the majority of human cases in the country.

The preparation of a pre-pandemic vaccine candidate must be coupled with the attenuation of the pathogenic potential of its parental virus strain in ferrets, chickens, and chicken embryos. We set out to characterize whether, in fact, Anhui/PR8 virus could be used as a safe and effective pre-pandemic candidate vaccine seed virus. The WHO has developed scientific guidance for the development of seed viruses.³⁵ The Anhui/PR8 reassortant virus demonstrated high-yield characteristics when grown in eggs, which is an important feature needed for the successful manufacturing scale-up process. This reassortant virus contains six internal genes from the egg adapted PR8 donor virus (PB2, PB1, PA, NP, M, and NS) which is responsible for the high-yield characteristic. It also contains the HA and NA genes from A/Anhui/01/2005 virus and sequence analysis confirmed that the HA of Anhui/PR8 virus has a modified cleavage site, lacking a four basic amino acid sequence necessary for systemic virus replication. As a result, Anhui/PR8 virus resembles low pathogenic avian influenza viruses in its cleavage motif and displayed a strict dependence on trypsin supplementation for the formation of plaques on CEF cells. In agreement with these *in vitro* studies,

the Anhui/PR8 virus was not lethal to chicken embryos unless it was inoculated at very high doses.

The survival of 100% of the chickens inoculated with Anhui/PR8 virus and the absence of clinical signs indicated that the reassortant virus is not pathogenic for chickens according to the classification of avian influenza established by the World Organization for Animal Health (OIE).^{25,35} In addition, the reassortant virus safety test in ferrets showed that none of the infected animals developed substantial signs of disease over the 14 d.p.i. experimental period. All of the above data indicate that the Anhui/PR8 virus meets the criteria to be classified as a low pathogenic avian influenza virus that complies with national and international safety standards.

Importantly, the Anhui/PR8 virus elicited an antibody response in ferrets that could not distinguish the reassortant virus from wild type virus and *vice versa*, indicating that the antigenic properties of the parental A/Anhui/01/2005 virus were maintained in the reassortant virus. In addition, antisera against Anhui/PR8 virus also neutralized other clade 2.3.4 H5N1 viruses (Table 1). All the manipulations required for the cloning of A/Anhui/01/2005 HA and NA genes, and its rescue and subsequent propagation in eggs were performed in dedicated facilities, using qualified Vero cells certified for vaccine use and reagents under a quality management system free of animal raw materials.³⁵ In addition, quality tests showed that the Anhui/PR8 virus was not pathogenic to chicken embryos (Table 5), did not display significant amounts of plasmid DNA or sterility issues (data not shown). These findings support the proposed use of this virus for development of seeds and production of inactivated pre-pandemic vaccine for use in clinical trials.

Acknowledgements

The authors would like to thank Aleksandr Lipatov and Li-Mei Chen for critical reading of the manuscript and Xiu-Feng Wan for suggestions. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

Author disclosure statement

The authors state that there are no competing financial interests.

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