



Generation of an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses

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Abstract

In the face of disease outbreaks in poultry and the potential pandemic threat to humans caused by the highly pathogenic avian influenza viruses (HPAIVs) of H5N1 subtype, improvement in biosecurity and the use of inactivated vaccines are two main options for the control of this disease. Vaccine candidates of influenza A viruses of H5N1 subtype have been generated in several laboratories by plasmid-based reverse genetics with hemagglutinin (HA) and neuraminidase (NA) genes from the epidemic strains of avian viruses in a background of internal genes from the vaccine donor strain of human strains, A/Puerto Rico/8/34 (PR8). These reassortant viruses containing genes from both avian and human viruses might impose biosafety concerns, also may be do if C4/F AIV would be a live attenuated vaccine or cold-adaptive strain vaccine. In order to generate better and safer vaccine candidate viruses, we genetically constructed attenuated reassortant H5N1 influenza A virus, designated as C4/F AIV, by plasmid-based reverse genetics with all eight genes from the avian strains. The C4/F AIV virus contained HA and NA genes from an epidemic strain A/Chicken/Huadong/04 (H5N1) (C4/H5N1) in a background of internal genes derived from a low pathogenic strain of A/Chicken/F/98(H9N2). The reassortant virus was attenuated by removal of the multibasic amino acid motif in the HA gene by mutation and deletion (from PQRERRRRKKR↓G to PQIETR↓G). The intravenous pathogenicity index (IVPI) of C4/F AIV virus was 0, whereas that of the donor virus C4/H5N1 was 3.0. The virus HA titer of C4/H5N1 in the allantoic fluid from infected embryonated eggs was as high as 1:2048. The inactivated vaccine prepared from the reassortant virus C4/F AIV-induced high HI titer in vaccinated chickens and gave 100% protection when challenged with highly pathogenic avian influenza virus of H5N1 subtype.

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1. Introduction

Influenza virus is a globally important respiratory pathogen which causes a high degree of morbidity and mortality in humans and animals annually [1]. Avian influenza A viruses bear all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes. Since the late 1990s, some of avian influenza A viruses have transmitted directly from birds to humans, such as H5 subtype highly pathogenic avian influenza (HPAI) [2–4]. H5N1 (HPAIV) is continuously undergoing antigenic change to escape the host's acquired immunity, so

the unprecedented magnitude of the bird flu prevail in some countries of the world [5,6]. During 2003–2006, a highly pathogenic avian influenza A (H5N1) virus caused poultry disease in Asian countries and infected many people [7], most of these individuals had close contact with poultry. Therefore, aside from active surveillance measures that monitor the transmission of these viruses to poultry, the development of an H5N1 AIV vaccine may be the best strategy to protect poultry against the threat of an H5N1 influenza epidemic or pandemic. The effectiveness of the vaccines that are currently available for influenza depends primarily on the antigenic “match” of the circulating viruses with the strains used for vaccination. However, not all viruses that are closely related are suitable for vaccine production, some grow poorly in eggs. To overcome the classical methods of

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selecting reassortants and time-consuming multiple passaging of viruses, a plasmid-based system is developed to rapidly generate infectious influenza viruses. Generally, the reassortants are generated by plasmid-based system combined with the high virus yield of the laboratory strain PR8 and the virus expressing of the glycoprotein of the currently circulating strain [8–10]. Influenza PR8 is a laboratory strain of influenza, that is also known to be neurovirulent and lethal for mice. For a big poultry industry in a country, there needs inactivated preparations on a large scale, there might be potential risk to vaccine recipients. Moreover, these reassortant viruses would be live attenuated vaccine or cold-adaptive strain vaccine and capable of inducing broad mucosal and systemic responses. Therefore, we generated an attenuated reassortant H5N1 AIV vaccine candidate with all eight genes from avian viruses by reverse genetics, that is, the vaccine seed virus derived a modified H5 HA from the H5N1 AIV virus, the N1 NA from the H5N1 AIV, and the internal genes of A/Chicken/Shanghai/F/98 (H9N2) that would confer the high growth phenotype in eggs. The generation and phenotypic properties of the reassortant C4/F virus were described.

2. Materials and methods

2.1. Viruses and cells

A/Chicken/Huadong/04 (H5N1) (C4/H5N1) and A/Goose/Huadong/04 (H5N1) (G4/H5N1) viruses isolated in China in 2004, and A/Chicken/Shanghai/F/98 (H9N2) (F) AIV isolated in Shanghai in Mainland China in 1998 [11]. They were propagated in the allantoic cavity of 10-day-old embryonated eggs of specific-pathogen-free (SPF) origin. Transfections were carried out in COS-1 (African green monkey kidney fibroblast-like) cells (Invitrogen Corp., Carlsbad, CA) maintained in Dulbecco's minimal essential medium with 0.3% bovine serum albumin (Invitrogen Corp., Carlsbad, CA), and HEPES (Invitrogen Corp., Carlsbad, CA). All experiments using infectious pathogenic avian influenza viruses (H5N1, H9N2), including the work with animals, were conducted using biosafety level 3+ containment procedures.

2.2. Plasmids

All eight genes of influenza F were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from viral RNA and cloned into pHW2000 [12], following the plasmids were constructed pHW201-PB2, pHW202-PB1, pHW203-PA, pHW204-HA, pHW205-NP, pHW206-NA, pHW207-M and pHW208-NS [12].

2.3. H5 HA plasmid

The H5 HA gene was amplified in two parts by RT-PCR from viral RNA of C4/H5N1, using primers designed to

delete the multibasic amino acid cleavage site as indicated in Fig. 1. The PCR products were cloned into pUC19 in a tri-molecular ligation reaction. The multiple basic amino acids of the HA cleavage site (PQRERRRKKR↓G) of C4/H5N1 that are associated with the virulence of the H5 avian influenza virus in chickens and mammals were changed into PQIETR↓G, a characteristic of low pathogenicity avian influenza viruses by site directed mutagenesis [8]. The insert was then subcloned into pHW2000 [13].

2.4. Transfection

The C4/F (H5N1) reassortant virus was generated by plasmid-based reverse genetics, cotransfecting eight plasmids (HA and NA derived from C4/H5N1 viruses, and the remaining six gene transcription plasmids from F strain) into COS-1 cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) [13]. About 24 h after transfection, the cells were scraped into the media and injected into embryonated eggs. The harvest from the first passage in eggs was injected into additional eggs. The transfectant viruses (C4/F and F) were identified in the allantoic fluid from the second egg passage, respectively. After 20 serial passages and adaption in embryonated chicken eggs of specific-pathogen-free (SPF) origin, the growth properties of the C4/F reassortant were confirmed.

2.5. Pathogenicity studies in chickens

Groups of eight 4-week-old SPF white leghorn chickens were inoculated with wild-type C4/H5N1, C4/F, or F transfectant viruses at a standard dose, 0.2 ml of a 1:10 dilution of stock virus, by the intravenous (i.v.) route, respectively [14]. Oropharyngeal and cloacal swabs were collected for virus isolation on day 3 postinoculation (p.i.) or on the day of death in chickens that died before day 3, and virus isolation titers were determined. All surviving chickens were euthanized and bled, and sera were tested for evidence of seroconversion by HI tests on day 14 p.i.

2.6. Pathogenicity studies in BALB/c mice

50% mouse lethal doses (MLD₅₀) of the H5N1 wild-type C4/H5N1, the C4/F and F transfectant viruses were determined by inoculating groups of six 6–8-week-old female BALB/c mice intranasally (i.n.) with serial 10-fold dilutions of the viruses [8]. Mice were checked daily for signs of disease for 21 days p.i.

To investigate the ability of the viruses to replicate in different organs, group of eight 6–8-week-old female BALB/c mice were infected i.n. with $50 \times 10^6 \mu\text{l}$ 50% eggs infectious dose (EID₅₀) of each virus and four mice from each group were euthanized on days 4 and 6 p.i., respectively. Lung, tracheal and brain tissues were harvested and homogenized in 1 ml phosphate-buffered saline (PBS)

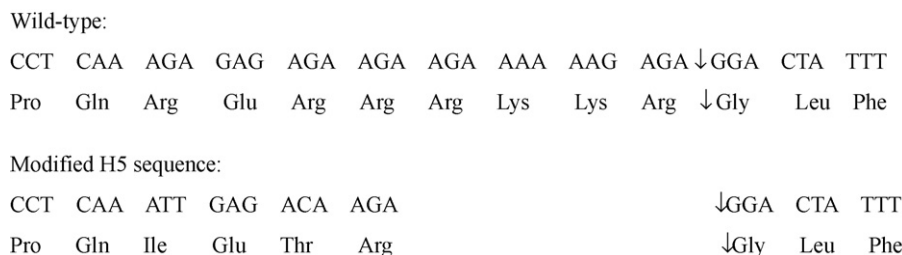


Fig. 1. Modified the cleavage site of HA of C4/H5N1. The arrow indicates the site at which HA1 and HA2 are cleaved.

and the tissue homogenates were titrated in eggs, as described previously [15].

2.7. Immunogenicity and efficacy of formalin-inactivated vaccines in chickens

Virus was inoculated into the allantoic cavities of 10-day-old embryonated eggs and harvested after 72 h incubation at 35 °C. To detect the content of the HA protein, we run the SDS-PAGE of the freshly harvested allantoic fluid and scanned the gel using the GeneSnap software of Bio Imaging Systems (SYNGENE), and protein of the HA band (which has been confirmed by Western blotting analysis using the H5 HA DNA vaccine immunized SPF chicken antisera in the preliminary analysis) was quantified with the GenTools software using the standard BSA protein. Then, the virus was inactivated by adding 0.2% formalin (v/v) and kept at 37 °C for 24 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of formalin-treated allantoic fluid in embryonated eggs. The inactivated allantoic fluid was emulsified in two parts of paraffin oil (Hangzhou Oil Refining Company, Hangzhou, China) (v/v), which is currently used commercially as adjuvant for veterinary vaccine production. The HA protein content in the final vaccine preparation is 9.6 µg/ml [16].

Three groups of ten 7-day-old white Leghorn SPF chickens were injected intramuscular (i.m.) with 0.3 ml PBS or 0.3 ml formalin-inactivated vaccine preparations (containing 3.1 µg HA protein). Sera were collected randomly from 10 chickens of each group 3 weeks postvaccination (pv) for HI antibody detection using the WHO standard method. Ten chickens from each group were challenged with 100 EID₅₀ of the homologous virus C4/H5N1 or heterogeneous virus G4/H5N1 intranasally at 3 weeks pv. Oropharyngeal and cloacal swabs of the chickens were collected on day 4 postchallenge for virus titration, and chickens were observed for disease signs and death for 2 weeks after challenge.

3. Results

3.1. Generation of the C4/F reassortant virus and confirmed it in vitro growth properties

The HA gene of H5N1 viruses encoded a multibasic amino acid motif in the connecting peptide, which is associated

with the systemic spread of viruses and high pathogenicity in chickens [2]. The HA gene was mutagenized as indicated in Fig. 1 to alter the multibasic cleavage site seen of C4/H5N1 virus to that of or low pathogenic avian influenza A viruses.

The plasmids were cotransfected in COS-1 cells that contained the HA and NA of C4/H5N1 and the six plasmids encoding the internal genes of F strain. The genotypes of the transfectant viruses (C4/F) were confirmed by restriction fragment length patterns and partially sequencing the F-derived genes and sequencing the full-length H5 HA and N1 NA genes. The sequences of the HA and NA were identical to those of the corresponding plasmids. The HA titers of the transfectant viruses C4/F was found in the majority of infected eggs to be 1280–2048 and infectivity titer of 8.0 log₁₀ ELD₅₀/ml in the allantoic cavity of embryonated eggs. The recombinant C4/F virus was shown by HI assay to be antigenically identical to the parental virus C4/H5N1. After 20 serial passages and adaption in embryonated chicken eggs of SPF origin, the C4/F reassortant showed good growth property and stable HA titer as high as 1:2048. The attenuated virus with an intravenous pathogenicity index (IVPI) of zero in chickens was steady.

3.2. Pathogenicity and replication in chickens

The data presented in Table 1 indicated that the removal of the multibasic amino acid cleavage site in the HA resulted in loss of pathogenicity of the C4/F transfectant virus for chickens. When the viruses were administered to groups of eight chickens in i.v., the H5N1 wild-type viruses were lethal in 100% of chickens, with a mean time to death of 1.5 days, all chickens shed virus in the oropharynx and cloaca, with mean titers of 6.4 ± 0.25 log₁₀ EID₅₀/ml and 3.8 ± 0.52 log₁₀ EID₅₀/ml, respectively. In contrast, the C4/F and F transfectant viruses were not lethal for chickens, few of the birds inoculated with the F transfectant virus shed low titers of virus in the oropharynx and cloaca, and all birds seroconverted, virus shedding was detectable in Oropharyngeal or cloacal swabs of chickens that received C4/F i.v., and seroconverted with mean titers of 4.2 ± 1.51 log₁₀ EID₅₀/ml and 2.5 ± 2.20 log₁₀ EID₅₀/ml, respectively.

3.3. Pathogenicity and replication in BALB/c mice

As shown in Table 2, the C4/H5N1 viruses could replicate in the tracheae, lungs and brains of mice and were

Table 1
Pathotyping and replication of intravenously administered the C4/F transfectant and the two parent viruses in chickens^a

Virus	Mortality (death/total)	Virus isolation from swabs ^b			Seroconversion ^c
		Oropharyngeal (shedding/total (titers))	IVPI	Cloacal (shedding/total (titers))	
F	0/8	5/8 ($\leq 1.0 \pm 0$) ^d	0	1/8 ($\leq 1.0 \pm 0$) ^e	8/8
C4/H5N1	8/8	8/8 (6.4 ± 0.25) ^d	3	8/8 (3.8 ± 0.52) ^e	8/8
C4/F	0/8	8/8 (4.2 ± 1.51) ^d	0	8/8 (2.5 ± 2.20)	8/8

^a Groups of chickens were infected intravenously (i.v.) with 0.2 ml 1:10 dilution of stock viruses.

^b Oropharyngeal and cloacal swabs were collected for virus titration on day 3 after inoculation. Virus titers were expressed as the mean \pm S.D. \log_{10} EID₅₀/ml from eight chickens.

^c Chickens were euthanized 2 weeks after infection and sera were harvested.

^d $P < 0.05$ compared with titer in corresponding swabs from C4/H5N1, C4/F or F inoculated chickens.

^e $P < 0.05$ compared with titer in corresponding swabs from C4/H5N1 or F inoculated chickens.

lethal for BALB/c mice (MLD₅₀ 3.5 \log_{10} EID₅₀/50 μ l). The donor of the internal genes, F, was a mildly pathogenic avian influenza virus strain in chickens [11], virus replication was not detected in the tracheae, lungs and brains of mice inoculated with the F virus. The titers of C4/F viruses in the tracheae and lungs were $1.1 \pm 1.78 \log_{10}$ EID₅₀/ml and $2.1 \pm 1.89 \log_{10}$ EID₅₀/ml, respectively, while the titers of C4/H5N1 viruses were $4.2 \pm 1.59 \log_{10}$ EID₅₀/ml and $5.3 \pm 4.60 \log_{10}$ EID₅₀/ml, respectively. So the C4/F transfectant virus replicated to lower titers than the H5N1 wild-type in trachea and lungs of mice, not isolated from brain, and not lethal for BALB/c mice. The virus was detected at a very low titer on day 6 in the brains of two of four mice inoculated with the wild-type H5N1 virus (C4/H5N1).

3.4. Immunogenicity and protective efficacy of a formalin-inactivated C4/F virus vaccine

Single intramuscular (i.m.) dose (0.3 ml formalin-inactivated vaccine preparations containing 3.1 μ g HA protein) of formalin-inactivated vaccines were prepared from the wild-type C4/H5N1 and the C4/F. To evaluate the immune response, we vaccinated 7-day-old chickens with one dose. Three weeks after immunization, the C4/F vaccine-induced higher HI titers to C4/H5N1 and G4/H5N1 than did wild-type

C4/H5N1. The C4/F formalin-inactivated vaccine protected chickens from subsequent challenge with 100 ELD₅₀ of C4/H5N1 virus, as did the formalin-inactivated wild-type C4/H5N1 vaccine. Under these conditions, none of the vaccinated birds developed disease signs, but 1 out of 10 vaccinated chickens with C4/F vaccine shed virus in their trachea and feces, and 6 out of 10 vaccinated birds with C4/H5N1 vaccine shed virus from their tracheae and 2 of 10 from their feces. All chickens, which were mock vaccinated with PBS in control group, died within 1.5–3 days (Table 3).

4. Discussion

Measures for the control of emerging and reemerging H5N1 influenza include the use of inactivated vaccines and improvement in biosecurity. Development of vaccines against H5 HPAIV pose several problems not previously encountered in the generation of influenza vaccine candidates. First, HPAI viruses are lethal to embryonated eggs, which limits growth in a high titer. Second, the multibasic amino acid motif at the HA cleavage site is believed to contribute to the virulence of these viruses in humans as well as in domestic poultry. Plasmid-based reverse genetics [10,13,18–20], is a powerful

Table 2
Pathogenicity and replication of the C4/F transfectant and the two parent viruses in BALB/c mice

Virus ^a	Day	Shedding/total (titer)			MLD ₅₀ ^c (\log_{10} EID ₅₀)
		Tracheae ^b	Lungs ^b	Brain ^b	
C4/H5N1	4	3/4 (4.2 ± 1.59) ^d	3/4 (5.3 ± 4.60)	2/4 (2.5 ± 0.51)	3.5
	6	2/4 ($\leq 1.0 \pm 0$)	2/4 (1.3 ± 0.58)	1/4 ($\leq 1.0 \pm 0$)	
F	4	0/4	0/4	0/4	0
	6	0/4	0/4	0/4	
C4/F	4	1/4 (1.1 ± 1.78) ^d	3/4 (2.1 ± 1.89)	0/4	0
	6	1/4 ($\leq 1.0 \pm 0$)	2/4 ($\leq 1.0 \pm 0$)	0/4	

^a 10^6 EID₅₀ of each virus was administered in 50 μ l i.n.

^b Virus titers were calculated by the method of Reed and Muench [16], and are expressed as \log_{10} EID₅₀ mean \pm S.D. of organs harvested from four mice. The lower limit of detection of virus in organs was 1.0 \log_{10} EID₅₀/ml.

^c 50% mouse lethal doses were determined by intranasal (i.n.) inoculation of groups of six mice with serial 10-fold dilutions of virus stock over a range from 10^{-1} to 10^{-10} .

^d $P < 0.05$ compared with corresponding titers in C4/H5N1 or C4/F inoculated mice.

Table 3
Immunogenicity and protective efficacy of a formalin-inactivated vaccine prepared from the C4/F transfectant virus

Immunogen ^a	HAI antibody titer against ^b		Protection against challenge ^c			
	C4/H5N1	G5/H5N1	Swabs virus titer ^d		Percent survival ^e	
			Oropharyngeal	Cloacal	C4/H5N1	G5/H5N1
C4/H5N1	208	None	7/10 (<1)	2/10 (<1)	100 ^f	100 ^g
C4/F	512	64	1/10 (<1)	1/10 (<1)	100 ^f	100 ^g
PBS	0	0	ND (dead)	ND (dead)	0 ^f	0 ^g

ND: not done.

^a Groups of 7-day-old SPF chickens were vaccinated with 0.3 ml of the vaccine preparations or PBS i.m.

^b Serum samples were collected 3 weeks after inoculation vaccine.

^c Chickens were challenged i.m. 3 weeks after inoculation vaccine with 100 EID₅₀ of C4/H5N1 or G5/H5N1 wild-type (wt) viruses.

^d Virus titers determined on day 4 p.i. are expressed as the mean log₁₀ EID₅₀/ml ± S.D. of 10 chickens per group.

^e Percentage survival at 21 days for groups of 10 chickens that were challenged with the lethal C4/H5N1 or G5/H5N1 virus.

^f *P* < 0.001 compared with the titer in PBS group.

^g *P* < 0.001 compared with the titer in PBS group.

tool to generate ideal avian-human (AH) reassortant influenza vaccine candidates [9,21–26]. The AH reassortant influenza A vaccines were developed using the high virus yield of the laboratory strain PR8 with the expression of the glycoprotein of the currently circulating strain avian influenza virus H5N1. The removal of the multibasic amino acid motif in the HA gene, associated with high pathogenicity in chickens, and the new genotype of the H5N1/PR8 transfectant virus, attenuated the virus for chickens and mice without altering the antigenicity of the HA. We believed that PR8 used a donor of internal genes of human vaccine may be suitable, because the virus derives from human origin and reduce the potential risks for cross-species transmission. Whereas for the vaccine of avian influenza, PR8 can replicate in mice [8], even if it used as inactivated preparation, would require heightened biocontainment to protect workers and the environment, which would be prohibitive for large scale vaccine production [18–27]. So in this study, we developed an attenuated H5N1 avian influenza virus vaccine with all eight genes from avian viruses, a reassortant C4/F converted the basic amino acids of HA gene seen in the C4/H5N1 viruses to the sequence motif seen in avirulent avian influenza viruses, was generated by reverse genetics with two surface genes from C4/H5N1 and the remaining six genes from F, which is not pathogenic and replicate for mice and low pathogenic for chickens. After 20 serial passages and adaption in embryonated chicken eggs of SPF origin, the C4/F reassortant showed good growth characteristic with the HA titer as high as 1:2048. The attenuated virus with an IVPI of zero in chickens was stable, and especially replicated to lower titers than the H5N1 wild-type in the trachea and lungs of mice, and was not lethal for chicken and mice. This data indicated that although the C4/F transfectant virus replicated in the trachea and lungs of mice, the attenuation phenotypes in the HA and the accompanying genotype are associated with a loss of virulence for chicken and mice. On the other hand, the reassortant viruses would used as a live attenuated or cold-adaptive strain vaccine candidate that would induce broad mucosal and systemic responses, and increase biosafety in human [28].

The pathogenicity of the virus and the sequence of its HA gene were not changed when the virus was passaged in embryonated chicken eggs. To determine the protective efficacy of the inactivated vaccine made of C4/F reassortant, 7-day-old white Leghorn SPF chickens were immunized. The HI titers were higher in chickens immunized with the inactivated vaccine of C4/F than those immunized with the inactivated vaccine of wild-type C4/H5N1. All chickens immunized with either C4/F or C4/H5N1 inactivated vaccine were completely protected from wild-type H5N1 or G4/H5N1 challenge 21 days pv. So C4/F with formalin-inactivated vaccines that was sufficiently related to the wild-type H5N1 viruses to elicit cross-protective immunity was sought as a candidate surrogate avian vaccine virus.

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