Original Article

H5N1 Avian Influenza Pre-pandemic Vaccine Strains in China*

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Abstract

Objective To prepare the 4 candidate vaccine strains of H5N1 avian influenza virus isolated in China.

Methods Recombinant viruses were rescued using reverse genetics. Neuraminidase (NA) and hemagglutinin (HA) segments of the A/Xinjiang/1/2006, A/Guangxi/1/2009, A/Hubei/1/2010, and A/Guangdong/1/2011 viruses were amplified by RT-PCR. Multibasic amino acid cleavage site of HA was removed and ligated into the pCIpolI vector for virus rescue. The recombinant viruses were evaluated by trypsin dependent assays. Their embryonate survival and antigenicity were compared with those of the respective wild-type viruses.

Results The 4 recombinant viruses showed similar antigenicity compared with wild-type viruses, chicken embryo survival and trypsin-dependent characteristics.

Conclusion The 4 recombinant viruses rescued using reverse genetics meet the criteria for classification of low pathogenic avian influenza strains, thus supporting the use of them for the development of seeds and production of pre-pandemic vaccines.

Key words: Influenza; H5N1; Pre-pandemic; Vaccine strains

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INTRODUCTION

Avian influenza viruses can not efficiently infect humans due to their viral genetic determinants and host factors[1-2]. The first human case caused by the highly pathogenic avian influenza H5N1 crossing the species barrier was reported in Hong Kong in 1997. Many of those infected with H5N1 have a rapid progression of viral pneumonia that leads to acute respiratory distress syndrome and death. Humans infected with H5N1 present with a high pharyngeal viral load and viral RNA is detected in their rectum and blood. The serum chemokine and cytokine levels are high in fatal cases[3-4]. HPAI H5N1 viruses continue to evolve at both the genetic and antigenic levels and the resultant HA gene diversity updates the periodic H5N1 virus classification. Twelve new H5N1 clades have been identified in recent years[5]. Furthermore, WHO reported that 345 of the confirmed 584 humans infected with H5N1 died with a cumulative case-fatality rate of 59% (30%-80%).

At the antigenic level, H5N1 viruses show a intra- and interclade cross-reactivity[6]. Despite the cross reactivity of certain H5N1 viruses, it is difficult to predict whether a vaccine strain protects against a strain of a different clade (or even sometimes of the same clade). Although human to human

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transmission of H5N1 is rare, viral evolution increases the possibility of an influenza pandemic in humans. If a H5N1 strain becomes readily transmissible between humans, it likely results in an influenza pandemic. It was recently reported that avian H5N1 influenza virus can transmit from ferret to ferret, an experimental animal model of human to human transmission[7-8].

Vaccine and antiviral agents play a key role in mitigating H5N1 pandemic. The representative H5N1 candidate vaccine viruses developed by the WHO Global Influenza Program are one of the overall global strategies for pandemic. The WHO has recommended that a pre-pandemic vaccine stockpile and 16 H5N1 vaccine seed viruses should be available with 4 in production or pending[9]. In recent years, plasmid-based reverse genetics has been applied in the rational development of influenza virus high growth reassortants. Typically 6:2 reassortants have been created in which the HA and NA segments are derived from a variety of wild-type human and avian viruses, including H1N1, H3N2, H5N1, and H5N3 human and avian viruses with the remaining 6 segments from A/PR8/34[10-12].

In China, the first fatal case of human H5N1 infection was confirmed in Anhui Province in 2005[13], and the number of human cases increased to 38 in June 2010. Of these 38 human cases, 28 belong to clades 2.4, and 1 belongs to clade 2.2, and 2 belong to clades 2.3.2[14]. Based on their antigenic characteristics, A/Xinjiang/1/2006 (clade 2.2), A/Guangxi/1/2009 (clade 2.3.2.1), A/Hubei/1/2010 (clade 2.3.2.1), and A/Guangdong/1/2011 (clade 2.3.2.1) have been proposed as candidate vaccine viruses since 2006. In this study, 4 recombinant viruses were rescued using reverse genetics. Reassortants were created in which the HA and NA segments were derived from A/Xinjiang/1/2006, A/Guangxi/1/2009, A/Hubei/1/2010, and A/Guangdong/1/2011 with the remaining 6 segments from A/PR/8/34. The HA gene was modified to remove the multibasic amino acids in the cleavage site. The yield, avirulence and equivalent antigenicity of the reassortant viruses were evaluated compared with the parent viruses.

MATERIALS AND METHODS

Cells, Viruses, and Serum

H5N1 human isolate A/Xinjiang/1/2006 (XJ), A/Guangxi/1/2009 (GX), A/Hubei/1/2010 (HB), and A/Guangdong/1/2011 (GD) viruses were propagated in embryonated chicken eggs aged 10 d The human 293T cell line used for rescue of reassortant virus from plasmids was cultured in a DMEM containing 10% FBS. Rg_A/Indonesia/05/2005 (Indo05), Rg_A/turkey/Turkey/1/05, Rg_A/common magpie/HongKong/5052/2007 (CM5052), A/Whooper swan/Hokkaido/4/2011, A/Anhui/1/2005 (AH/1) viruses, and anti XJ, anti GX, anti HB, antiIndo05, antiAH/1, and antiCM5052 sera were provided by the Chinese National Influenza Center, the National Institute for Viral Disease Control and Prevention (IVDC).

Plasmid DNA

The plasmid containing PR8 internal genes and pCIpolI vector were gifts from the United States Centers for Disease Control and Prevention (US CDC).

Isolation of Viral RNA, Rreverse Transcription, PCR Amplification, and Sequencing

The vRNA was extracted from infected embryonated chicken eggs containing 140 μL allantoic fluid with a RNeasy kit (Qiagen, Hilden, Germany) and eluted into 40 μL of H_2O, Thrity-five RT-PCR cycles were performed, each for for 50 min at 50 °C, for 15 min at 94 °C, for 30 s at 94 °C, for 30 s at 55 °C, and for 3 min at 72 °C, according to its manufacturer's instructuctions. PCR product was sequenced with the rhodamine dye-terminator cycle sequencing ready reaction kit using AmpliTaq DNA polymerase FS (Perkin-Elmer Applied Biosystems, ABI) according to its manufacturer's instructions.

Mutagenesis and Cloning

The HA1 and HA2 genes of XJ, GX, HB, and GD were amplified by PCR to remove the multibasic amino acids at the cleavage site and digested with SapI (NEB, USA) to form the blunt ends for ligation into the pCIpolI vector. The NA gene was amplified by PCR, digested with SapI and ligated into the pCIpolI vector. The internal PA, PB1, PB2, NP, NS, and M genes of A/PR/8/34 were ligated into the pCIpolI vector. Plasmids were sequenced and compared with those generated from the wild-type virus. Only clones that exactly matches the parental virus sequence were used for virus rescue.

Virus Rescue

The plasmids containing modified HA and NA genes of XJ, GX, HB, GD, and the 6 internal genes of

PR8 (1 μg of each) were incubated for 30 min with Trans-IT (Mirus, USA) in Opti-MEM medium (Invitrogen, Carlsbad, USA) before added (dropwise) to 293T cells prepared on day 1 and cultured in a 6-well plate. The cell density was 4×10^5/per well. The transfectant mixture was added onto the 293T cell surface followed by 1 mL Opti-MEM medium containing 1 μg trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK). Forty-eight hours after transfection, the cell supernatant was collected and inoculated in 10-day specific pathogen free embryonated eggs (Merial, China) for propagation of rescued viruses at 35 °C for 48 h. The allantoic fluid was collected and virus titer was measured by HA titer. The resuced viruses were aliquote and frozen at -80 °C.

Trypsin Dependence Assay

Viruses in primary chicken embryo fibroblasts (CEF) were examined by plaque assay. The CEF were prepared on day 1 and cultured in a 6-well plate with a density of 1×10^6. On the next day, confluent cell monolayers in the 6-well plate were infected with 10-fold dilution of virus in a total volume of 0.2 mL of PBS for 1 h at 35 °C. The cells were washed once with PBS and covered with an overlay of MEM containing 0.9% agar and 1 μg/mL TPCK. The plates were incubated at 37 °C in a atmosphere containing 5% CO_2 for 3 d with the overlays removed and the cells fixed with methanol for 30 min at room temperature (22-25 °C). The fixative was then aspirated and the cells were washed with PBS and stained with 1% (g/v) crystal violet for 10 min. The excess stain was washed with PBS and air-dried. The number of plaques was calculated and the size of plaques was measured as previously described[15].

Chicken Embryo Lethality Test

Ten-day-old chicken embryos were obtained from Merial Company. The viruses were diluted with PBS in a $\log_{10}$ dilution and 0.1 mL of each virus was injected into the allantoic cavity of embryos. Eight eggs were incubated with each virus at 35 °C for 48 h with dead embryos idendified daily.

Antigenic Characterization by Hemagglutination Inhibition Assay

Hemagglutination inhibition (HI) was assayed as previously described[16]. Briefly, sera treated with receptor destroying enzyme (RDE. Denka Seiken, Tokyo, Japan) overnight at 37 °C were inactivated by incubation at 56 °C for 30 min and two-fold serially diluted in v-bottom microtiter plates, starting at 1:10. An equal volume of virus, adjusted to 4 HA units /25 μL was added to each well. After 30 min of incubation at RT, 50 μL of 0.5% (vol/vol) turkey erythrocytes suspension was added to each mixture and incubated. The inhibition of hemagglutination at the highest serum dilution was considered as the HI titer of the sera.

RESULTS

HA Gene Mutagenesis

Four H5N1 viruses showed different multibasic amino acids located in the HA gene cleavage site (Table 1). The strategy was to amplify the HA1 and HA2 separately. HA1 and HA2 were ligated into the pCIpolI vector. Recombinant plasmids with modified HA gene were sequenced and the correct clones were selected (Figure 1).

Table 1. Deletion of The Virulence-inducing Cleavage Motif in The hemagglutinin Segment of Different H5N1 Viruses

Virus	Amino Acid Sequence at Cleavage Site
XJ wt	LRNSPQGE***RRRKK***RGLFGAIAG
GX wt	LRNSPQRE***RRRRK***RGLFGAIAG
HB wt	LRNSPQRE***RRRK***RGLFGAIAG
GD wt	LRNSPQIE***RRRRK***RGLFGAIAG

Note. Bold Italic represent cleavage site sequence.

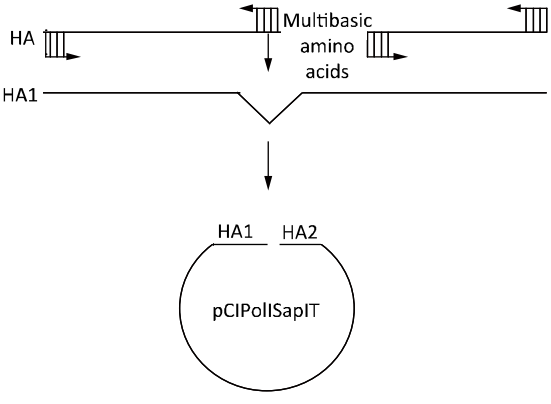

Figure 1. The strategy of removing the multi-basic amino acids located at cleavage site.

Rescue Mutagenesis Viruses

The modified HA gene combined the NA of XJ, GX, HB, GD, and other genes from PR8 virus were rescued on 293T cells and passaged onto embryonated eggs. Viral nucleic acid was extracted and the genes were sequenced. The HA gene sequence showed that the cleavage site was removed. The sequences of amino acids in NA and internal genes are similar to those of parental and PR8 viruses. The virus yields detected by HA titer showed that the 4 reassortant viruses replicated quite well with their HA titer higher than 512 (Table 2).

Antigenic Analysis of Wild Type and Reassortant Viruses

The HI assay is commonly used to measure the reactivity of HA. The antigenicty of XJ, GX, HB, and GD wild type viruses was assayed using the 6 antisera-raised toward viruses Indo05 (clade 2.1), AH1 (clade 2.3.4), CM5052, GX, HB (clade 2.3.2.1), and XJ (clade 2.2). The HI assay indicated that anti XJ sera reacted with XJ wild type and XJ recombinant virus with a HI titer of 1280 and 640 respectively. The HI titer showed 4-fold differences between XJ and Rg A/turkey/Turkey/1/05. The antigenicity of GXrg, HBrg, and GDrg viruses was detected using anti CM5052, anti GX and anti HB sera respectively. GX wild virus reacted well with antiCM5052 sera with a HI titer of 320. The antigenicity of GX was closely related with that of CM5052. HB wild type virus reacted with antiCM5052 with a HI titer of 80 while HB reacted poorly with anti GX sera with a HI titer of 40, the GD wild type virus reacted poorly with antiCM5052 and antiHB sera with a HI titer <10 and HI=40 respectively, indicating that the antigenicity of GX, HB, and GD is different. All wild type viruses and their own reassortant virus showed the same antigenic characteristic (Table 3).

Table 2. Hemagglutination Titers of Reassortant Viruses

Virus	Passage History	HA titer
XJ Rg	C1E2*	1:2048
GX Rg	C1E2	1:512
HB Rg	C1E2	1:512
GD Rg	C1E2	1:1024

Note. *C1E2 means recombinant viruses were rescued on 293T cell (C1), then passage two generations on embryonated eggs (E2) .

Chicken Embryo Lethality Test

Twenty-four hours after the chicken embryos aged 10 d were inoculated with reassortant and wild type viruses, viable embryos were observed. The wild type viruses were highly lethal to chicken embryos and killed all eggs while all the embryos survived after inoculation with reassortant viruses (Table 4).

Table 3. Antigenic Characterization of Reassortant Viruses

Viruses	Clade	Ferret Antisera					
		clade					
		2.1	2.3.4	2.3.2.1	2.3.2.1	2.3.2.1	2.2
		Indo05	AH/1	CM5052	GX	HB	XJ
Rg_A/Indonesia/05/2005 (IBCDC-RG2)	2.1	20480	160	20	160	40	320
Rg_A/turkey/Turkey/1/05 (NIBRG-23)	2.2	1280	<10	160	20	20	80
A/Anhui/1/2005	2.3.4	2560	320	<10	80	<10	320
Rg A/common magpie/HongKong/5052/2007	2.3.2.1	320	<10	160	80	40	NA
A/Whooper swan/Hokkaido/4/2011	2.3.2.1	160	<10	80	40	<10	NA
GX wt	2.3.2.1	320	<10	320	320	80	320
GX Rg		320	<10	160	320	80	320
HB wt		160	<10	80	40	40	40
HB Rg		160	<10	80	40	40	20
GD wt		80	<10	<10	40	<10	20
GD Rg		160	<10	<10	20	<10	20
XJ wt	2.2	160	20	20	40	<10	1280
XJ Rg		160	40	20	40	<10	640

Formation of Trypsin-dependent Plaques

Highly pathogenic H5N1 viruses were replicated while reassortant viruses did not form plaques in the absence of trypsin. Wild type viruses formed plaques and produced a similar amount of PFU in the absence or presence of TPCK, indicating that reassortant viruses lack of virulence determinant (Table 5).

DISCUSSION

HPAI H5N1 virus continues to evolve and is a greater public health threat if they acquire the ability to cause sustained and widespread human to human transmission leading to the next influenza pandemic. Epidemiology study showed that 31 H5N1 cases identified in China revealed diverse genotypes which are consistent with those identified in poultry outbreak or in live poultry markets. Live poultry markets are an important source of human H5N1 infection in China. Live poultry markets, backyard poultry breeding, and domestic poultry farms are the potential sources of human H5N1 infection, and visiting live poultry markets has been identified as an important factor in such infections in China[14,17]. Antigenic profiling with MAbs demonstrated that H5N1 virus has undergone rapid antigenic changes since 2003[18]. H5N1 virus isolated from human cases in China showed the genetic and antigenic diversity. The most isolated H5N1 viruses belong to clade 2.3.4, indicating that the antigenicity of XJ virus is different from that of representative virus rg A/turkey/Turkey/1/05 (clade 2.2). Although GX, HB, and GD viruses belong to the same clade 2.3.2.1, the antigenicity of GX virus is similar with that of the representative virus CM5052, while the antigenicity of HB and GD is significantly different from that of GX and representative virus. The antigenicity of the 4 viruses is the representative of the clade 2.2 and clade 2.3.2.1 viruses in China. It is thus important to prepare the candidate virus vaccine strains based on the antigenicity characteristics of H5N1 virus isolated in China.

Pandemic influenza is challenging to its vaccine. At present, several technologies are under development for pandemic vaccines. Reverse-genetics technology allows the generation of safe vaccine strains with its known properties and advantages of rapid vaccine preparation[19]. HPAI H5N1 viruses can kill the embryonated eggs that are used for vaccine production with a good viral antigen titer. Furthermore, use of nonatteuated HPAI virus as a vaccine seed virus necessitates the BSL-3 containment. Reverse genetics used to remove the multibasic amino acids at the cleavage site is employed to generate a recombinant virus candidate with the HA and NA of the putative vaccine candidate on a PR8 backbone[20]. The 4 ecombinant candidate vaccine strains constructed in this study are non-virulent to eggs with a high titer. In addition, the best recombinant viruses clones were achieved as a vaccine seed strain for increasing their growth stability. The 4 recombinant viruses can remove the multibasic amino acids of HA and can thus be cultured under BSL-2 level.

Table 4. Virulence Assessment in Chicken Embryos

Virus	Survival
XJ wt	0/8
GX wt	0/8
HB wt	0/8
GD wt	0/8
XJ Rg	8/8
GXRg	8/8
HB Rg	8/8
GD Rg	8/8

Table 5. Trypsin-dependent Plaque Formation

Virus	Plaque			
	With Trypsin		Without Trypsin	
	PFU/mL	Diameter (mm)	PFU/mL	Diameter (mm)
XJ wt	5.2×10^7	1.5±0.1	5.5×10^7	1±0.25
XJ Rg	4.8×10^6	2.0±0.2	n/a	n/a
GX wt	1.3×10^7	2.0±0.15	1.3×10^7	2.1±0.1
GX Rg	1.5×10^7	2.3±0.15	n/a	n/a
HB wt	4.0×10^6	0.8±0.2	4.0×10^6	0.8±0.23
HB Rg	3.2×10^6	1.2±0.2	n/a	n/a
GD wt	9×10^7	1.8±0.15	9.3×10^7	1.6±0.05
GD Rg	8×10^7	2.0±0.15	n/a	n/a

Note. Data indicated as Mean±SD.

Reverse genetics is also used to generate agricultural H5N3 vaccines that can induce anti-HA antibodies and prevent death of chickens. A candidate H5N1 reverse-genetics vaccine assessed according to the A/Vietnam/1203/04 virus is underway in United States and Europe. Clinical trials of candidate H5N1 vaccines have been either initiated or completed, including rg-A/Vietnam/1203/2004 (VN), rg-A/Indonesia/05/2005(ID), and rg-A/Anhui/1/2005 (AH). Immune strategies are selected to provide broader cross protection[21]. Multiple-clade adjuvanted vaccine containing rg-A/Vietnam/1203/2004 (VN), rg-A/Indonesia/05/2005 (ID), and rg-A/Anhui/2005 (AH) elicits strong immune response and is cross protective. Vaccine strains derived from clade 1 viruses under different stages of clinical trials, show a limited cross-clade reactivity[22-23].

In this study, the antigenicity of recombinant viruses was similar with that of wild type viruses. However, the antigenicity of XJ virus differed from the same clade representative virus A/turkey/Turkey/1/05. The antigenicity of GX, HB, and GD viruses belong to the same clade 2.3.2.1 was different, indicating that the antigenicity of clade 2.3.2.2 virus is diverse in China. Cross protection of the 4 candidate vaccine strains needs to be further assessed in animal models and humans.

The H5N1 antigenic divergence gives a new challenge to pandemic preparation. Protective immunity of a vaccine is effective against viruses that are antigeniticaaly matched with other vaccine strains. In China, A/Anhui/1/2005 is selected as the representative clade 2.3.4 and Anhui/PR8 reassortant virus has been developed against the circulating clade 2.3.4 virus[24]. Along with HA evolution, different H5N1 antigenicity triggers the need of new matched vaccine strains. The 4 representative viruses isolated in China can meet the criteria for low pathogenic avian influenza, thus supporting the use of H5N1 virus for development of seeds and production of pre-pandemic vaccines.

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