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High-yield production of a stable Vero cell-based vaccine candidate against the highly pathogenic avian influenza virus H5N1

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ABSTRACT

Highly pathogenic avian influenza (HPAI) viruses pose a global pandemic threat, for which rapid large-scale vaccine production technology is critical for prevention and control. Because chickens are highly susceptible to HPAI viruses, the supply of chicken embryos for vaccine production might be depleted during a virus outbreak. Therefore, developing HPAI virus vaccines using other technologies is critical. Meeting vaccine demand using the Vero cell-based fermentation process has been hindered by low stability and yield. In this study, a Vero cell-based HPAI H5N1 vaccine candidate (H5N1/YNVa) with stable high yield was achieved by reassortment of the Vero-adapted (Va) high growth A/Yunnan/1/2005(H3N2) (YNVa) virus with the A/Anhui/1/2005(H5N1) attenuated influenza vaccine strain (H5N1delta) using the 6/2 method. The reassorted H5N1/YNVa vaccine maintained a high hemagglutination (HA) titer of 1024. Furthermore, H5N1/YNVa displayed low pathogenicity and uniform immunogenicity compared to that of the parent virus.

1. Introduction

The HPAI H5N1 virus has been circulating in Europe and Asia for more than a decade, spreading to more than 60 countries, and infecting 570 humans with 335 fatalities thus far [1]. Although reports of human-to-human HPAI H5N1 transmission are rare, its high lethality has raised considerable concern worldwide [2–4]. To control and prevent a possible HPAI H5N1 virus pandemic, it is critical to develop a rapid and large-scale vaccine-manufacturing technology. Currently, most commercial vaccines against influenza viruses, including the HPAI H5N1 vaccines, are mainly produced in chicken embryos [5,6]. While this method has been used for more than 50 years, there are several well-recognized limitations to this system in countering a possible HPAI virus pandemic. First, the global capacity of eggs for vaccine production is sufficient to protect only a small percentage of the worldwide population [7,8]; second, as chickens are highly susceptible to HPAI virus infection, the egg supply is inherently at risk [9]; third, this system is inharmonious with the advantages of current biotechnology [10,11]. An alternative is the cell-based fermentation process [12]. This system has many advantages over the egg-based method, as it is not dependent on the availability of chicken embryos, and has the potential to provide a large-scale, high-quality, inexpensive, and stable vaccine [13,14].

Vero cells have been licensed as a cell-based vaccine-production substrate by the World Health Organization (WHO) [15]. While useful for the development of an inactivated vaccine, this system is rarely used for attenuated HPAI H5N1 vaccine production owing to the unstable or low yields [16,17]. Thus, many enterprises have not adopted this convenient and fast production technology. To produce an effective live virus vaccine, several obstacles need to be addressed, including attenuating the virulence of the wild type strain, improving the yield of the attenuated virus, and determining the immunogenic effectiveness against ancestral wild type viruses.

We isolated a seasonal influenza A H3N2 virus, A/Yunnan/1/2005(H3N2) (YNVa), which could be amplified with high titer in Vero cells by industrial fermentation [18]. In this study, we replaced the hemagglutinin (HA) and neuraminidase (NA) genes of (YNVa) with those of the HPAI H5N1 virus A/Anhui/1/2005(H5N1) by reverse genetics technology, of which the HA1 and HA2 cleavage site was deleted to avert the possible pathogenicity aroused by the wild type HA protein. The reassortant H5N1/YNVa showed high titer production and stable replication

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ability in Vero cells, attenuated pathogenicity in chickens, and effective immunogenicity in mice.

2. Materials and methods

2.1. Virus strains and cell lines

The HPAI virus, A/Anhui/1/2005(H5N1), belonging to the 2.3.4 clade, was originally isolated from a fatal Chinese case [19]. The H5N1delta attenuated virus, provided by the US Centers for Disease Control (CDC, Atlanta, USA), of which attenuation was achieved by deleting multiple cleavage sites on HA [20], was propagated in chick embryos in a biosafety level 2 enhanced (BSL2+) facility. A/Yunnan/1/2005Va(H3N2), isolated from a patient in the Yunnan Province of China, was propagated in 10-day-old chick embryos and then transferred to Vero cells. This virus, termed YNVa, maintained a high titer in Vero cells, with more than 1024 HAU, for tens of passages [18]. This Vero cell-based high-yield H3N2 strain, YNVa, was stored at the Institute of Medical Biology, Chinese Academy of Medical Sciences (CCTCC NO: V200514). In this study, a 25-passage Vero-adapted virus was used as the backbone.

Vero cells were obtained from the European Collection of Cell Cultures (ECACC; code: 03129010), 293T human embryonic kidney cells were from the American Type Culture Collection (ATCC), and primary chicken embryo fibroblasts (CEFs) were obtained from the China CDC. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). DMEM/F12 (pH 7.0–7.2) containing 1% bovine serum albumin (BSA), 100 U/ml penicillin G, 100 μg/ml streptomycin, 2 mM L-glutamine, 25 mM HEPES buffer, and 1% L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was used for virus growth.

2.2. Virus recovery

Template viral RNA was extracted from H5N1delta and YNVa using the QIAamp viral RNA extraction kit (Qiagen, Valencia, CA). The PB2, PB1, PA, NP, M, and NS genes of YNVa and the HA and NA genes of H5N1delta were reverse-transcribed using a reverse transcription kit (Biovisualab Ltd, Shanghai, China). The cDNAs were amplified by HiFiFast DNA Polymerase (Biovisualab Ltd, Shanghai, China) using primers (PB2 forward: 5′-TATTGGTCTCAG GGAGCAAAAGCAGGTC-3′, PB2 reverse: 5′-ATATGGTCTCGTATTAGT AGAAACAAGGTCGTTT-3′; PB1 forward: 5′-TATTCGTCTCAGGGAGC AAAAGCAGGCA-3′, PB1 reverse: 5′-ATATCGTCTCGTATTAGTAGAAA AAGGCATTT-3′; PA forward: 5′-TATTGGTCTCAGGGAGCAAAAGCA GGTAC-3′, PA reverse: 5′-ATATGGTCTCGTATTAGTAGAAACAAGGTA CTT-3′; HA forward: 5′-TATTCGTCTCAGGGAGCAAAAGCAGGGG- 3′, HA reverse: 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′; NP forward: 5′-TATTCGTCTCAGGGAGCAAAAGCAGGGTT-3′, NP reverse: 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTATTTTT-3′; NA forward: 5′-TATTGGTCTCAGGGAGCAAAAGCAGGAGT-3′, NA reverse: 5′-ATATGGTCTCGTATTAGTAGAAACAAGGAGTTTTTT-3′; M forward: 5′-TATTCGTCTCAGGGAGCAAAAGCAGGTAG-3′, M reverse: 5′-ATATCGTCTCGTATTAGTAGAAACAAGGTAGTTTTT-3′; NS forward: 5′-TATTCGTCTCAGGGAGCAAAAGCAGGGTG-3′, NS reverse: 5′-ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT-3′) with the restriction enzyme sites for insertion into the bidirectional transcription system pHW2000 (provided by Dr. Hoffmann E, Memphis, TN) [21,22]. After confirming the sequence of all inserts, the correct plasmids were used for virus recovery.

Reassortant H5N1/YNVa was achieved by transfecting six plasmid DNAs encoding the internal protein genes of YNVa and two plasmids encoding the surface antigen genes of HA and NA from H5N1delta into 293T cells using Lipofectamine ™2000 (Invitrogen, CA) [23]. After 48 h, the transfected cells and supernatants were harvested for reassortant virus analysis.

2.3. Virus amplification in Vero cells

After removing the growth medium, Vero cell monolayers were washed three times with PBS, and inoculated with H5N1delta, H5N1/YNVa, or YNVa in 75 cm^2 flasks for a minimum of 30 min, up to 1 h at 37 °C. Next, infected monolayers were incubated at 33 °C with 20 ml virus growth medium and observed daily for cytopathic effects (CPEs). The cell supernatant was harvested from each flask when at least 75% of the cell monolayer exhibited CPE. After centrifuging the supernatant at 3000 rpm for 15 min at 4 °C, the supernatant was dispensed into 2 ml aliquots in sterile 2 ml cryovials and stored at −80 °C for further use.

2.4. Plaque assay on CEF cells with or without trypsin

CEF cells were grown on six-well culture plates in DMEM supplemented with 10% calf serum. After inoculation with serial dilutions of the virus stocks, monolayer cells were overlaid with 1.6% agarose in 2 × DMEM without serum, with or without 1 μg/ml TPCK-treated trypsin. Plates were further incubated for 72 h, then stained with 0.3% crystal violet to visualize plaques.

2.5. Chicken embryo lethality test

Ten-day-old chicken embryos were inoculated in the allantois with 0.1 ml 1:10 dilution of each virus preparation with known infectious titers. The virus dose that caused death in 50% of embryos was calculated by the method of Reed–Muench [24] and recorded as the median chicken embryo lethal dose (CELD50).

2.6. HA quantification

Virus samples were diluted two-fold in 0.1 M PBS serially, quantified with a standard HA assay using an equal volume of a 1% erythrocyte suspension (chicken) [16], and incubated in a V-shaped microtiter plate for 30 min at room temperature. The single radial immunodiffusion technique (SRID) assay was performed as previously described [25]. The antigen reagent for the single radial diffusion assay of A/Anhui/1/2005 influenza virus hemagglutination (National Institute for Biological Standards and Control [NIBSC] code: 07/290) and the influenza antiserum A/Anhui/1/2005(H5) (NIBSC code: 07/338) were provided by NIBSC (Hertfordshire, England).

2.7. Hemagglutination inhibition assay (HAI)

Aliquots of 25 μl of the two-fold serially diluted immunized mice antisera were incubated with 25 μl influenza virus at 37 °C for 1 h followed by incubation with 50 μl 1% erythrocyte suspension (chicken) at room temperature for 30 min. The HAI titer was defined as the highest serum dilution that inhibited hemagglutination.

2.8. Pathogenicity of H5N1/YNVa in chickens

All animal experiments were conducted in accordance with the Guidelines for Animal Experiments described and approved by the Institute of Medical Biology Chinese Academy of Medical Sciences and operated under BSL2+ conditions. To assess the pathogenicity of H5N1/YNVa, groups of eight 4-week-old specific-pathogen-free Plymouth white rock chickens were inoculated with the H5N1/YNVa and H5N1delta at a standard dose $10^{8.0}$ EID50 by the intravenous route (i.v.) and observed for 14 days.

2.9. Safety and immunogenicity studies of vaccine in mice

To evaluate the safety and immunogenicity of H5N1/YN, 4- to 6-week-old female BALB/c mice (20 in each group) were immunized with one dose of 0.2 ml 15 μg HA unit of formalin-inactivated [26] H5N1/YNVa and H5N1delta virus by subcutaneous injection. Weight changes were recorded daily at the same time. Sera were collected after 28 days for the HAI test.

2.10. Statistics

Significant differences were evaluated using a two-tailed Fisher's exact test (SPSS, release 12.1; SPSS Inc., Chicago, IL). Differences were deemed to be statistically significant at $p < 0.05$.

3. Results

3.1. Confirmation of the reassortant H5N1/YNVa virus

The reassortant virus H5N1/YNVa was obtained using the 6/2 method, in which the six backbone fragments PB2, PB1, PA, NP, M, and NS were derived from YNVa [18], whereas the two surface antigens, HA and NA, were from H5N1delta. To confirm the reassortant virus, HAI was first used to determine whether the antibody against H5N1delta could block the hemagglutination caused by the H5N1/YNVa virus with high specificity. Together with the sequencing, the reassortant virus was confirmed.

3.2. Growth kinetics of H5N1/YNVa in Vero cells

To determine the growth kinetics of the H5N1/YNVa virus in Vero cells, H5N1/YNVa and the two parent viruses, YNVa and H5N1delta, were propagated in Vero cells simultaneously. The YNVa Vero-adapted strain displayed a steady HA titer in Vero cells, maintaining an HA titer at around 938.7 ± 73.9 to 1280 ± 0.0 throughout the 10 passages (Fig. 1). The HA titer of H5N1/YNVa was 213.0 ± 73.9 at passage 1, which increased to 1024 ± 0.0 at passage 5, decreased slightly to 938.7 ± 0.0/2.0 at passage 6 and 7, and increased to 1194.7 ± 147.8 at passage 10 (Fig. 1). The HA titer of the H5N1delta virus gradually decreased from 981 ± 73.9 at passage 1 to 512 ± 0.0 at passage 2, and remained nearly undetectable from passage 3 to 10 (Fig. 1).

These results show that the growth kinetics of the H5N1/YNVa and H5N1delta are dramatically different, and that the H5N1/YNVa obtained a similar ability for high-yield growth from its ancestor, YNVa.

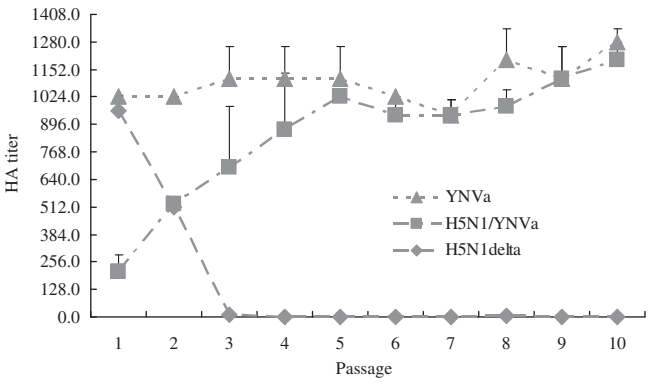

Fig. 1. Growth kinetics of H5N1/YNVa in Vero cells. H5N1/YNVa, YNVa, and H5N1delta were subjected to 10 continuous passages in Vero cells. The viral titer was detected at each passage by hemagglutination analysis.

Table 1
Plaque formation on CEF cells with or without trypsin.

Viruses	With trypsin		Without trypsin	
	PFU/ml	Diameter (mm)	PFU/ml	Diameter (mm)
H5N1/YNVa	$2.6 \times 10^{8.3}$	2–3	<1	NA
H5N1delta	$4.3 \times 10^{8.1}$	2–3	<1	NA

NA, not applicable.

Table 2
Virulence assessment of H5N1/YNVa in chicken embryo and chicken.

Virus	CELD50	Mortality death/total
H5N1/YNVa	$10^{5.6}$	0/10
H5N1delta	$10^{5.1}$	0/10

CELD50, 50% chicken embryo lethal dose. Infectious titer of 10^8 viruses were used for mortality assessment.

3.3. Plaque assay on CEF cells with or without trypsin

The H5N1delta virus is dependent on trypsin for HA maturation, displaying a genetic modification common to low-pathogenicity H5N1 viruses [27]. In this study, plaque assays, performed on CEF cells in the presence or absence of trypsin, were used to confirm the low pathogenicity of H5N1/YNVa. In the presence of trypsin, the H5N1/YNVa and H5N1delta formed plaques on CEF cells (at $2.6 \times 10^{8.3}$ and $4.3 \times 10^{8.1}$ PFU/ml, respectively), with a plaque diameter of around 2–3 mm (Table 1). In the absence of trypsin treatment, neither virus formed plaques on CEF cells (PFU/ml < 1). These results show that H5N1/YNVa cannot grow without trypsin.

3.4. Pathogenicity of H5N1/YNVa in chicken embryo and chicken

Ten-day-old chicken embryos were inoculated with serially diluted titers of H5N1/YNVa and H5N1delta. The CELD50 for H5N1/YNVa was $\geqslant 10^{5.6}$ PFU/ml, and that of H5N1delta was $\geqslant 10^{5.1}$ at 48 h after inoculation. Further, when chickens ($n = 10$) were inoculated with $10^{8.0}$ of H5N1/YNVa or H5N1delta, all chickens remained healthy throughout the 14-day observation period with no mortalities (Table 2). These results demonstrate that H5N1/YNVa has a low pathogenicity similar to that of H5N1delta.

3.5. Safety and immunogenicity of H5N1/YNVa in mice

To investigate the safety and immunogenicity of H5N1/YNVa, 4- to 6-week-old female BALB/c mice (20 in each group) were

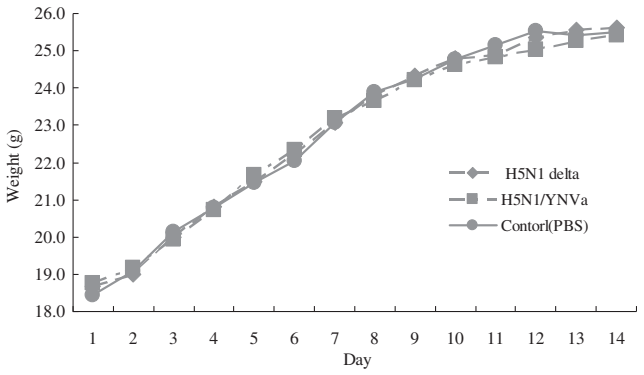

Fig. 2. Pathogenicity of H5N1/YNVa in mice. Inoculation was performed on day 1 using 20 mice in each group. The weight of the mice was recorded daily.

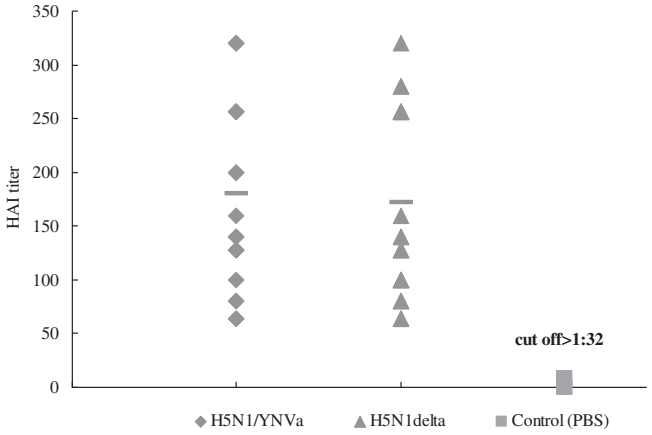

Fig. 3. Immunogenicity of H5N1/YNVa in mice. Twenty-eight days after inoculation, the sera of all mice were sampled for hemagglutination inhibition (HAI) analysis. HAI tests were performed in microtiter plates with 1% chicken red blood cells. Titers were recorded as the reciprocal of the lowest dilution of antibodies that inhibited hemagglutination.

immunized with one dose of (0.2 ml, 15 μg HA) formalin-inactivated H5N1/YNVa and H5N1delta virus by subcutaneous injection. Mice weight and behavior were recorded and observed daily for 14 days; as shown in Fig. 2, the body weights of the two groups were very similar to that of the control group. The weight of the H5N1/YNVa group increased from 18.8 ± 0.5 g at day 1 to 25.4 ± 1.5 g at day 14, and the weight of the H5N1delta group increased from 18.7 ± 0.5 at day 1 to 25.6 ± 1.4 at day 14 (Fig. 2).

On day 28, sera of all mice were sampled for antibody analysis. The sera from both the H5N1delta- and H5N1/YNVa-immunized mice inhibited hemagglutination with a mean titer of 172.2 ± 86.6 and 179.8 ± 88.2, respectively (Fig. 3).

These results demonstrate that H5N1/YNVa has a safety and immunogenicity profile similar to that of H5N1delta.

4. Discussion

Since recommended by the WHO as a virus vaccine-production substrate, Vero cells have been successfully employed in vaccine production. Vero cell-based manufacturing processes have been used for large-scale manufacturing of both seasonal and pandemic strain influenza vaccines, and clinical trials showed that a Vero cell-derived trivalent influenza vaccine is effective and safe [28,29]. Although there is much benefit to cell fermentation, developing appropriate influenza vaccine candidates is difficult due to the growth stability of the virus [30]. In this report, we utilized the well-characterized high-yield seasonal H3N2 virus, YNVa, as the backbone to improve the cell adaptability of the hybrid H5N1/YNVa. A series of experiments demonstrated that H5N1/YNVa has a growth ability similar to that of the YNVa (H3N2) parent, and an immunogenicity similar to that of the H5N1delta parent. Furthermore, the virulence of the reassortant H5N1/YNVa is low. Our work resolved some barriers to cell-based manufacturing of attenuated vaccines.

There are several reports regarding the reassortment of seasonal H3N2 and HPAI H5N1, but they mainly focused on the possible reassortment of the contemporary viruses and the relative virulence [31–33]. Our study concentrated on the utilization of the high-yield backbone viral component in Vero cells and the antigens displayed. Our data show that YNVa is qualified for this purpose, suggesting that we could promptly produce a large-scale vaccine in response to a pandemic due to HPAI viruses. Prior studies demonstrated highly pathogenic viral strains derived from the HPAI H5N1 reassorted with seasonal H3N2. In this study, the HA of HPAI H5N1 was attenuated by modification at the HA1/HA2 cleavage site [20]. The reassorted H5N1/YNVa virus had low virulence, and no atavistic heredity emerged during the virus-recovery process and cell-adaptive passage. Reverse genetics could also provide an attenuated HPAI virus vaccine strain for the chicken embryo-based manufacturing process. However, in this study, we established a cell-based method for recovering stable attenuated virus in 293T cells and amplifying the candidate in Vero cells directly.

Tseng et al. screened a Vero-adapted H5N1 vaccine strain, Vero-15, by serial passages and plaque purifications of the NIBRG-14 vaccine virus in Vero cells [34]. The candidate strain, having the H1N1 strain backbone, was derived from the reassortment between the A/Vietnam/1194/2004 (H5N1) virus and the egg-adapted high-growth A/PR/8/1934 virus [34]. In this study, we employed the backbone from YNVa, an H3N2 virus isolated and adapted by our group that showed Vero cell adaptability, and reverse genetics technology to design H5N1/YNVa. H5N1/YNVa displayed growth kinetics similar to those of YNVa. This result shows that H5N1/YNVa acquired the cell-adaptive ability from the YNVa strain.

This report mainly focused on obtaining high-yield production of a stable Vero cell-based vaccine candidate against the HPAI virus H5N1, current research put emphasis on the pathogenicity and growth kinetics of the H5N1/YNVa. We adopted the three main evaluation criterions of the European Agency for the Evaluation of Medicinal Products for the immunologic evaluation: the seroconversion rate for anti-HA antibodies was more than 40%, the mean geometric increase of anti-HA was more than 2.5, and the protective rate is more than 70% [35]. The inclusion of both the HA and NA glycoproteins in current influenza vaccines is thought to aid the production of valid immunity because these two envelope proteins have antagonistic functions in the viral release and entry processes. Of antibodies against NA, our previous study had reported that although anti-NA antibodies reduced viral amplification in the multiple-cycle infection assay, antibodies against NA boost influenza viruses infectivity [36].

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