RESEARCH PAPER

Preparation and evaluation of a novel, live, attenuated influenza H1N1 vaccine strain produced by a modified classical reassortment method

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ABSTRACT

Live attenuated influenza vaccine (LAIV)-based Vero cells could provide a better choice to control and prevent influenza virus infections. This study used the human influenza virus A/Yunnan/1/2005Vca(H3N2) (YN/05Vca) as a donor strain. YN/05Vca has a double phenotype of cold adaption (ca) and Vero cell adaption (va). The parental virus strain used was the wild-type A/Solomon Islands/3/2006 (H1N1) (SI/06wt). The study employed the modified classical reassortment method to generate a new virus strain. After co-infection of Vero cells, some different sub-types of the reassorted viruses were generated randomly. Then, the specific anti-serum (anti-YN/05Vca) could combine with and neutralize the donor virus, and the original parental virus could not grow in Vero cells at a low temperature until it was re-structured with the meaningful gene fragment from the donor virus in Vero cells. According to the plaques and RT-PCR results, a new monoclonal strain of Vero cell cold adaption virus was screened: SI/06Vca. After immunological and biological identification, this new strain virus could be used as a seed bank for LAIV, which has maintained surface antigenicity with SI/06wt. Consequently, this new Vero cell cold adaption virus SI/06Vca could be used for large-scale vaccine production with sufficient safety and efficacy, as confirmed by animal experiments with mice and ferrets.

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Introduction

The influenza virus is one of the important respiratory pathogens for humans, causing seasonal epidemics, and even pandemics, worldwide and inducing a high rate of mortality.¹ The influenza vaccine is a very effective measure to control influenza virus infection. Currently, there are three types of licensed seasonal influenza vaccines, including inactivated, live attenuated, and recombinant vaccines.² Inactivated influenza vaccines have been used clinically in humans for seven decades.³

Regarding live attenuated influenza vaccine (LAIV), there are more advantages. The vaccination pathway could occur through nasal immunization, which provides a convenient alternative for subjects and a similar route to natural infection. This route can elicit mucosal and cellular immunity in addition to humoral immunity, thus providing strong protective efficacy and long-lasting immunity.^{4,5} However, the challenge of developing LAIV is to generate one or more candidate virus strains with satisfactory attenuation phenotypes, as well as possessing high-yielding properties in the appropriate substrate.

Currently, three cold-adapted influenza A virus strains – A/Ann Arbor/6/60 (AA) (H2N2), A/Leningrad/134/17/57 (H2N2) and A/Leningrad/134/47/57 (H2N2) – and two B virus strains – B/Ann Arbor/1/66 ca and B/USSR/60/69 ca – have been developed to use as LAIV donor viruses to generate

appropriate seasonal influenza vaccines.^{6,7} However, there is no H1N1 sub-type strain. In 2003, the Food and Drug Administration (FDA) approved FluMist (MedImmune), by first using LAIV, which employed the cold adapted donor strains A/Ann Arbor/6/60 (H2N2) and B/Ann Arbor/1/66 ca.⁸

However, these LAIVs were only produced in chicken embryos and not in mammalian cells. Egg-based vaccines might have the potential risks of contamination and spread of uncertain disease, while the inadequate supply of chicken embryos could greatly limit the production of egg-derived vaccines, especially in the avian virus pandemic.⁹ Due to these factors, the World Health Organization (WHO) recommended the development of alternative influenza virus cultivation systems, specifically to explore promising mammalian cell culture lines (cell culture as a substrate for the production of influenza vaccines: Memorandum from a WHO meeting in 1995). Several cell lines are currently approved for cell culture-based vaccine production, such as the African green monkey (Vero) cell line (e.g., used for polio and rabies vaccines). However, there is currently no Vero cell-derived attenuated seasonal influenza H1N1 vaccine.

Most influenza vaccine viruses cannot grow in Vero cells, limiting the application of the Vero system for vaccine production. Actually, in recent years, only one cold adapted

donor strain in Vero cells, A/Singapore/1/57, was reported.¹⁰ However, there has been no further information about this vaccine reported. In our department, we screened an LAIV donor strain from hundreds of clinical samples, which we named A/Yunnan/1/2005Vca (H3N2). This donor influenza virus strain (YN/05Vca) has the double phenotype of cold adaption (ca) and Vero cell adaption (va). According to analysis of pathogenicity, it has an attenuation phenotype (att).

Regarding reassortant methods, the widely accepted reverse genetics approaches have allowed for the development of LAIV, but only for pandemic, and not seasonal, vaccines in China. In contrast, there are some specific uncontrollable factors in the usage of reverse genetics approaches. For example, the poly basic cleavage site of the agglutination gene base sequence is easy to be cut by restriction enzyme digestion.¹¹⁻¹³ Therefore, the classical manner of generating candidate vaccine viruses should be considered useful in China.

In this study, through co-infection on the same Vero cell plate well, we generated the Vero cell-based LAIV by modified classical reassortant methods. The sufficient efficacy and safety of the new vaccine strain supported our thesis.

Results

Confirmation of the reassortant SI/06Vca virus

Genome composition

After sequencing and BLAST searches for each of 8 gene fragments, it was found that its HA and NA gene fragments were from SI/06wt, and its other 6 gene fragments were from YN/05Vca. In contrast, in the process of screening the target monoclonal virus strain, other composition of gene fragments were not adopted in this study. As an additional analysis, the formation of genetic compositions as 6+2 was normal, but other formations were occasional or less normal by randomly selection of all of the 288 experimental strains of monoclonal virus on 25 6-well culture plates. The opportunities and research approaches of all the formations are listed in Table 1.

The gene sequence of the generated virus SI/06Vca was confirmed at the 1st, 10th and 20th generations. Furthermore, no meaningful changes in its 8 gene fragments were found in these serial passage processes. It had genetic stability at 25°C in Vero cells.

Table 1. The opportunities and research approach of all the formations.

Donor strain:YN/05Vca	Parental strain:SI/06wt	Opportunities	Approach
7 (PA, PB1, PB2, M, NS, NP, HA or NA)	1 (NA or HA)	Neutralized by antibody	Never found
6 (PA, PB1, PB2, M, NS, NP)	2 (NA and HA)	Normal (72%)	adopted
5 (PA, PB1, PB2, M, NS)	3 (NP, NA and HA)	Less normal (13%)	Discarded
4 (PA, PB1, PB2, M)	4 (NS, NP, NA and HA)	Occasional (10%)	Discarded
3 (PA, PB1, PB2)	5 (M, NS, NP, NA, HA)	Seldom (5%)	Discarded
2 (2 from PA, PB1, PB2)	6 of other	Not grow	Never found
1 (1 from PA, PB1, PB2)	7 of other	Not grow	Never found

Growth characteristics of SI/06Vca in Vero cell at 25°C

We cultured the reassortant virus (SI/06Vca), the donor virus (YN/05Vca) and the parental virus (SI/06wt) in Vero cells at 25°C, respectively, from the 1st to 18th passages in parallel testing. Then, we calculated their MOI in Vero cells, illustrating these changes in Fig. 1.

SI/06Vca could be stably serially passaged in Vero cells at 25°C with high-yield production, similar to YN/05Vca, especially from the tenth generation. However, the wild-type SI/06wt virus could not be serially passaged in Vero cells at 25°C, which declined from the 2nd generation.

As shown, the reassortant virus showed a 100-fold increase in growth titers by the 9th serial passage. The optimal culture system for the virus was sought. The important improvement was a pH value increase from 6.9 to 7.3, but the pH value of 6.9 was better for the YN/05Vca strain.

Growth characteristics of SI/06Vca at different temperature

There were three gradients of temperature – 25°C, 33°C and 39°C – in these experiments. Each experimental virus strain was cultured in its suitable cell substrate. In detail, YN/06Vca and SI/06Vca were cultured in Vero cells, while SI/06wt was cultured in MDCK cells. After growing the virus for 120 hours in cells, the medium was harvested for viral titer testing. If there were 100-fold changes (increase or decrease) in virus titers, it could be defined as two different phenotypes. According to the results in Table 2, we could determine whether each virus strain use had a ca or temperature sensitive (ts) phenotype.

These data indicated that both YN/06Vca and SI/06Vca had cold adaptability (ca) and temperature sensitivity (ts) phenotypes, but SI/06wt did not. The two ca virus strains could replicate efficiently at 25°C and 33°C, but they were restricted at 39°C. The results of SI/06wt were the opposite.

Biological safety assessment in mice

The condition of the mice was recorded and observed daily for 14 days, especially their conditions and weight changes after nasal immunization. No convulsions, shock, deaths or other serious adverse effects were recorded in the YN/06Vca and SI/06Vca groups. Flu-like symptoms in the upper respiratory system were observed within 3 days after each injection. Their weights increased and showed no significant differences between the Vca group and the PBS control group ($P > 0.5$), as shown in Fig. 2. After 14 days, the animals were in good condition. Their weights grew slowly and steady, illustrating that YN/06Vca and SI/06Vca were both safe for LAIV development.

However, in the SI/06wt group, the 8 mice were in worse condition. They lost more than 20% of their initial weight within 2 days after nasal injection. They showed varying degrees of serious adverse effects, including apathy, shivering, and eating and drinking less.

Under the supervision of the animal ethics committee of Peking Union Medical College, all 8 mice were euthanized with humanitarian methods on the 3rd day. The results suggested

Figure 1. Growth characteristics of SI/06Vca in Vero cells at 25°C, compared with YN/05Vca and SI/06wt, from the 1st to 18th passages in parallel testing, according to MOI testing.

that SI/06wt could not be used as a live attenuated vaccine directly.

Attenuated phenotype of new LAIV strain

We determined the 50 percent of minimal lethal dose (MLD₅₀) for SI/06Vca, SI/06wt and YN/05Vca in mice. The MLD₅₀ of SI/06wt was 10^{4.8}TCID₅₀, but the new LAIV strain, SI/06Vca, displayed a similar MLD₅₀ value to the donor strain YN/05Vca, which ranged from 10^{7.8}TCID₅₀ to 10^{7.9}TCID₅₀. The SI/06Vca strain was not as lethal in mice as the parental strain, which had an obviously attenuated phenotype.

In subsequent experiments, the virus replication levels were examined *in vivo* in mice. SI/06Vca and YN/05Vca replication produced high viral titers in mouse turbinates (from 10^{5.7}TCID₅₀/g to 10^{5.8}TCID₅₀/g) but low titers in the lungs of infected animals (from 10^{3.2}TCID₅₀/g to 10^{3.4}TCID₅₀/g). These tests were implemented on the third day after inoculation. Regarding the wild type virus strain SI/06wt, in contrast, its replication level was 10^{7.6}TCID₅₀/g in the turbinates and 10^{8.3}TCID₅₀/g in the lung. These results proved that the live attenuated virus successfully infected the animals. However, the LAIV strains could not achieve high replication in the animals' lungs, different from wild type, which illustrated that the LAIV virus could not cause dam-

age to lung cells. Therefore, the SI/06Vca strain was conducive to extensive use as a live attenuated vaccine.

Assessment of effective antigenicity by serum

We collected and separated the serum of mice after nasal immunization. The preparation process of serum was previously described.¹⁴ First, its HI titer was detected using SI/06wt (H1N1) and YN/05Vca (H3N2) virus strains. As shown in Table 3, we found that the serum by SI/06Vca injection had little cross-reaction with YN/05Vca (H3N2) virus (1:2) but a specific binding reaction with SI/06wt (H1N1) virus (1:2048). Regarding serum by YN/05Vca injection, the results were the opposite.

Micro-neutralization testing was performed to calculate the GMT of antibody titers. There was no significant difference between SI/06Vca and SI/06wt virus in its capability to stimulate antibodies ($P > 0.05$). By supplementation, their seroconversion rate could attain 100% in the SI/06Vca group experiment.

Evaluation of tissue penetration efficacy in ferrets

Doses of 10⁵TCID₅₀/mL, 10⁶TCID₅₀/mL and 10⁷TCID₅₀/mL of SI/06Vca were administered by nasal injection, with 500 μ L per ferret. After three days post-injection, we collected and separated their nasal mucosa tissue (NT), bronchus, lungs and brain to detect the deposited SI/06Vca virus. Using the homogenates of these tissues, we separated the supernatant by centrifugation at 4000 rpm/min for 20 min. Then, we seeded them on the Vero cells.

We observed the CPE and calculated the MOI value to assess the presence of live attenuated virus in different tissues. It was found that live attenuated virus SI/06Vca could reproduce in the nasal mucosa tissue and maintain a certain vitality. The production was not accompanied by a significant dose effect with original MOI.

Table 2. Growth characteristics of SI/06Vca at different temperature and its determination of ca and ts.

Virus strain	Mean virus titer $\pm$ SE (Log10TCID ₅₀ /mL) at indicated temperature			Determination of whether had these phenotype	
	25°C (n = 8)	33°C (n = 8)	39°C (n = 8)	ca	ts
SI/06wt	4.0 $\pm$ 0.03	8.0 $\pm$ 0.02	6.9 $\pm$ 0.02	No	No
SI/06Vca	6.8 $\pm$ 0.18	7.8 $\pm$ 0.03	3.5 $\pm$ 0	Yes	Yes
YN/05Vca	7.1 $\pm$ 0.08	8.4 $\pm$ 0.12	3.7 $\pm$ 0.07	Yes	Yes

Figure 2. The changes in mice's weights within 14 days after intranasal immunization with all of the virus strains used to evaluate their animal safety.

However, in bronchus tissues, a broken window effect was observed when the original dose of 10^7 TCID₅₀ was used. This dosage could induce almost six times the dose of 10^5 TCID₅₀ and 10^6 TCID₅₀. We could not detect live virus in the brain tissues of ferrets. All of the results of animal safety assessments suggested that the reassortant SI/06Vca under 10^7 TCID₅₀ had sufficient safety for a LAIV, as shown in Fig. 3.

Challenge experiment to verify its efficacy

All of the experimental ferrets vaccinated with SI/06Vca were fully protected against the SI/06wt infection at 10 times the MLD₅₀. There was an absence of signs of disease and limited weight loss after challenge, compared with the PBS control groups. In contrast, mock-vaccinated mice succumbed to challenge within 5 days post-challenge. All 12 of the ferrets were in good condition, except for some flu-like symptoms in the upper respiratory tract. Their behavior, spirit, eating and drinking were active or normal, consistent with the previous findings in mice.

Discussion

Currently, live attenuated influenza vaccines (LAIV) are allowed to be produced in embryonic chicken eggs. Regarding its positive aspects, LAIV is not subjected to some complex purification, inactivation and re-purification processes, as occurs with split or inactivated vaccines. LAIV could be new development direction for influenza vaccines.

However, regarding the negative aspects, using LAIV based on eggs might cause unfavorable reactions in some specific

populations due to allergy to albumin. The HA antigenic variation in eggs might be an unignored risk factor in vaccine production. In addition, according to the WHO requirements, only pathogen-free chick embryos can be used for the production of LAIV.¹⁵ There has been evidence that retrovirus genome segments can be found even in such embryos,¹⁶ not to mention the inadequate supply of eggs during influenza pandemics.

Thus, it is necessary to find a new basis for LAIV production. The use of the passage of mammalian cell lines to produce LAIV could largely overcome the above limitations on mass production. According to some scientific findings, Vero cells are an advanced choice for influenza vaccine because they have been licensed for other vaccines, such as polio and rabies.¹⁷

Although it has been shown that most influenza viruses can grow in Vero cells at low levels, improvements in culture technology and basic medicine studies could resolve this problem, if permitted by the WHO.¹⁸ Feasibility analyses of Vero cell-based influenza vaccines have been published in recent years.¹⁹⁻²¹

In our department, we screened a live attenuated influenza A virus YN/05Vca (H3N2), which could be serially passaged in Vero cells at 25°C, and its attenuated characteristics have been verified over the past decade, which were also analyzed in this study. However, the largest constraint is that its HA and NA immunology is inconsistent with the annual epidemic strain. Therefore, YN/05Vca could be used as a donor strain but not an original vaccine strain.

In this study, using YN/05Vca as a master donor strain, we generated a reassortant virus SI/06Vca, which carried the HA and NA gene segments from a wild-type seasonal influenza strain (SI/06wt) to assure the same antigenicity. The other 6 gene fragments were from YN/05Vca, with the formation of 6+2. After co-infection in Vero cells, we selected the SI/06Vca monoclonal by plaque and then sequenced its whole genome. Only the virus with formation of 6+2 was used in the subsequent study. Other formation viruses were discarded. In contrast, we indeed found formations such as 5+3 and 4+4.

We used the classical approach for the generation of a live-attenuated influenza virus vaccine candidate. Some scientists have found that the current state of the field involves the use of reverse genetics. Actually, the reverse genetics approach had

Table 3. The results of effective immunogenicity of serum by HI and GMT calculation.

Serum from different virus strains sources (N = 8)	HI titer for different subtype virus strains		GMT of each antibodies	t value	p value
	SI/06wt (H1N1)	YN/05Vca (H3N2)			
SI/06Vca (H1N1)	1:2048	1:2	182.4 ± 4.6	-0.16	0.8742
YN/05Vca (H3N2)	1:2	1:2048	179.7 ± 5.08

Figure 3. Detection of the MOI in different tissues immunized with SI/06Vca to assess the replication of live attenuated virus by different methods.

been employed for almost ten years in our department for influenza virus reassortment. However, experiments with some special strains could not generate new target reassortant strains successfully for a variety of reasons. For example, the HA gene was degraded by restriction endonucleases. There was unexplained base mutation in the HA or NA gene. The greater challenge came from HA antigenic changes, which caused the new strain to lose sufficient effects. This SI/06wt strain was not treated with the reverse genetics approach for this reason. Reviewing the development of reassortant approaches, we believe that the reverse genetics and classical reassortant approaches are equally helpful for the generation of new target reassortant viruses, according to necessity.

The reassortant SI/06Vca virus had lower pathogenicity than before reassortant without fatal infection. All of the subject mice and ferrets were in good condition after nasal injections. Furthermore, their weights increased, and there were no significant differences between the Vca groups and the PBS control group ($P > 0.5$). We could conclude that reassortant SI/06Vca had good animal safety, but SI/06wt should not be considered as a live attenuated vaccine directly because weight loss exceeded 20%.

In the virus tissue penetration efficiency experiments, we found that 10^7 TCID₅₀ was a threshold value of viral replication in the bronchi, while SI/06Vca could not up-link to infect the brain or down-link to infect the lungs.

By analyzing the serum of experimental animals, the results supported that SI/06Vca can stimulate the body to produce effective antigenicity with a low degree of cross-reaction for other sub-types. In contrast, the seroconversion rate could reach 100% with sufficiently high GMT, and in the challenge experiments, we had good harvest expected results, with less than ten times the MLD₅₀ of the wild-type virus.

These characteristics of the SI/06Vca virus met the attenuation criteria for live attenuated influenza virus vaccines,²² including clear genetic constitution, high-yield production in Vero cells at 25°C, a sufficient animal safety and effective antigenicity.

Furthermore, the used donor strain YN/05Vca and even the generated strain SI/06Vca might be used as donors for other genetic reassortant vaccine strains, maintaining Vero cell adaption and cold adaption in the future.

Our findings could be used in the local area of Yunnan to some degree. After consultation with the Department of Acute Infectious Diseases of Yunnan Infectious Disease Control and Prevention Center (CDC), we collected influenza epidemic information in Yunnan province over nearly a decade (from 2006 to 2016). It was found that many influenza epidemic strains, which had antigenic homology with A/Solomon Islands/3/2006 (H1N1), were multi-regional and long-term epidemics. For example, A/Weiyuan/16/2006 was epidemic from 2006 to 2007 in the northeast of Yunnan, A/Wuhua/3/2013 was epidemic from 2013 to 2014 in the central and eastern regions, A/Hongta/1/2014 was epidemic from 2014 to 2015 in the southern region, and A/Ruili/5/2015 was epidemic from 2015 to 2016 in the western region and caused one death of a 9-year-old boy. Due to the majority of the Yunnan Basin having the special climate of plateau season, the same or similar antigenic viruses with SI/06wt posed a high risk to the health of local residents, except those who received suitable vaccines from 2008 to 2012.

Over the next one to five years, it is uncertain whether other influenza viruses with this similar antigenicity could cause epidemics again because they did not use the similar HA vaccine in the Yunnan area. Therefore, this study could provide a useful choice to prevent this type of influenza virus infection in the next step, which is also the pursuit of the innovation team in Yunnan Province (2015HC027).

Limitations: In the ferret experiment, the only data shown are the virus titers in several tissues, again with the signs and symptoms of disease mentioned in the text. It would be informative to perform serology for these animals as well. For any work toward a vaccine candidate, a qualified cell line would have to be used. Therefore, the current donor virus would need to be re-created in such a cell line for clinical use. We should

undertake more recent work experiment reassortment with 2009 pdmH1N1 virus and H3N2 virus in the future.

Materials and methods

Virus

The donor strain used was A/Yunnan/1/2005Vca (H3N2), abbreviated YN/05Vca, which was isolated from a patient sample in the Yunnan Province of China. After culture in Vero cells, it was adapted to high-yield production by serial passage to improve growth. Additionally, its lower virulence was assessed by animal experiments, which were certified.²³ Importantly, this donor influenza virus strain (YN/05Vca) has the double phenotype of cold adaption (ca) and Vero cell adaption (va), and it was stored at the Institute of Medical Biology, Chinese Academy of Medical Sciences.

The parental strain used was wild-type A/Solomon Islands/3/2006 (H1N1) abbreviated SI/06wt, isolated from wild-type seasonal influenza virus. It was obtained from the National Institute for Biological Standard and Control (NIBSC, UK). The tissue culture infected dose (TCID₅₀) of each virus strain was detected according to the methods reported by Reed and Muench in 1938.²⁴

Cells and tissue culture

Vero cells were obtained from the European Collection of Cell Cultures (ECACC; code: 03129010). MDCK cells were obtained from the American Type Culture Collection (ATCC). We cultured these two strains of cells according to the supplied manuals. After culture for 48 h, the cells were infected by influenza virus using DMEM/F12 (Gibco) as a maintenance medium, which contained 0.1% bovine serum albumin (BSA), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 25 mM HEPES, and 1 µg/mL L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Washington Diagnostics, Freehold, NJ, USA).

Antibodies

Goat anti YN/05Vca serum was made by the Institute of Medical Biology. Briefly, after booster immunizations three times (once per month) of four adult rams, the serum was separated and treated with receptor destroying enzyme (RDE, Denka Seiken, Japan) to remove non-specific agglutination inhibitors, and it was heated to 56°C for 1 h to inactivate its complement.

The hemagglutination inhibition (HI) titer of this qualified anti-serum should be greater than 1:5120 for YN/05Vca but have no cross-activity with other sub-types (e.g., H1N1) in its quality evaluation process.

Reassortant virus in Vero cells

All of the reassortant virus-related operations were performed under bio-safety level three conditions, according to the requirements and guidelines of the Chinese Center for Disease Control and Prevention (CDC). The reassortant virus experiment used the classical method, which has been described previously.^{13,22} In this study, we needed to detect the multiplicity of infection

(MOI) of each virus strain at each stage on the MDCK or Vero cells if needed. The reassortant process was as follows.

10⁸TCID₅₀/mL of the SI/06wt virus and 10⁷TCID₅₀/mL of the YN/05Vca were mixed in 300 µL of phosphate-buffered saline (PBS). Then, the same well with the Vero cells was co-infected (in a 6-well plate) and repeated in 3 wells. Each independent strain and blank controls were created in the 3 other wells.

At the first stage, we cultured these virus at 33°C for 3 days and then harvested the reassortant virus. An equal volume of prepared antiserum was added to bind to YN/05Vca specifically, which was incubated at 33°C for 30 min for subsequent experiments.

At the second stage, the virus mixture was used to infect the same well with the Vero cells at 25°C for 7 days. Additionally, we added the prepared antiserum once every 48 hours with the same volume. There was an internal control with the independent strain of YN/05Vca. If the cytopathic effect (CPE) of Vero cells in the control well was observed, it was necessary to add antiserum once more. We repeated this operation three times in this second stage. Finally, it generated the reassortant virus in Vero cells, named SI/06Vca.

Identification of the virus seed SI/06Vca

Gene composition

RT-PCR methods were used to clone the 8 gene fragments of the donor strain (YN/05Vca), parental strain (SI/06wt) and newly generated strain (SI/06Vca). Each pair of primers for each fragment is listed in Table 4. The whole genome for the SI/06Vca virus was sequenced by Shanghai Biological Engineering Technology Services Ltd.

Then, we analyzed the gene composition of SI/06Vca by BLAST with two other strains for their 8 gene fragments. We were sure that its PB1, PB2, PA, M, NS and NP fragments were from the YN/05Vca, and its HA and NA fragments were from SI/06wt. This principle was the standard for selection of the monoclonal SI/06Vca strain. If not, other gene-types would be discarded.

Serially passaged in Vero cells at different temperature ranges

We seeded the 0.1 mL of SI/06Vca with 10⁷TCID₅₀/mL on Vero cells in the T₂₅ flask. When 80% CPE was observed, we

Table 4. Each pairs of primer for each fragmentation of influenza A virus.

Serial number	Primer name	Sequence of primer
1	PB2-F	TATTGGTCTCAGGGAGCAAAGCAGGTC
	PB2-R	ATATGGTCTCGTATTAGTAGAACAAGGTCGTTT
2	PB1-F	TATTCGTCTCAGGGAGCAAAGCAGGCA
	PB1-R	ATATCGTCTCGTATTAGTAGAACAAGGCATTT
3	PA-F	TATTCGTCTCAGGGAGCAAAGCAGGTAC
	PA-R	ATATCGTCTCGTATTAGTAGAACAAGGTACTT
4	HA-F	TATTCGTCTCAGGGAGCAAAGCAGGGG
	HA-R	ATATCGTCTCGTATTAGTAGAACAAGGGTGTTTT
5	NP-F	TATTCGTCTCAGGGAGCAAAGCAGGGTA
	NP-R	ATATCGTCTCGTATTAGTAGAACAAGGGTATTTTT
6	NA-F	TATTGGTCTCAGGGAGCAAAGCAGGAGT
	NA-R	ATATGGTCTCGTATTAGTAGAACAAGGAGTTTTTT
7	M-F	TATTCGTCTCAGGGAGCAAAGCAGGTAG
	M-R	ATATCGTCTCGTATTAGTAGAACAAGGTAGTTTTT
8	NS-F	TATTCGTCTCAGGGAGCAAAGCAGGGTG
	NS-R	ATATCGTCTCGTATTAGTAGAACAAGGGTGTTTT

harvested all of the virus culture medium with 10 ml. Then, we detected its MOI and seeded it for the next passage.

As a parallel test, we cultured the SI/06wt and YN/05Vca at 25°C, 33°C and 39°C, respectively. Furthermore, we cultured the original donor and parental strains under the same MOI, temperature and Vero cell basis. We should note that 0.4 µg/ml TPCK-trypsin was added once every 72 hours to help cleave its HA protein.

Biological safety evaluation in mice

The mass production of SI/06Vca in Vero cells at 25°C was implemented by cell factory. The live attenuated virus was purified by the Department of Key Laboratory of Kunming Medical University. According to its reports, the MOI of purified SI/06Vca was 10^8 TCID₅₀/mL.

A total of 24 female BALB/c mice at 8 weeks old were randomly divided into three groups. Each group (n = 8) received nasal injection (n.i.) with different virus strains (SI/06Vca, SI/06wt and YN/05Vca) after anesthesia by sodium pentobarbital (60–80 mg/kg). Titers were expressed as TCID₅₀. MLD₅₀, a dose required to kill 50% of mice, was calculated following the previously described methods for determination.²⁵

A medical observation was performed from 1 to 3 weeks after infection with one-tenth of the MLD₅₀. Weight changes were recorded daily. It required more care for the severe lost weight and even deaths of mice. Two mouse turbinates and lungs were collected in each group at three days post-infection, and these organs were used to examine the virus replication levels in different tissues. The mice were fed in strictly sterile, laminar flow, separate animal rooms, with a room temperature of 25°C and air humidity of 50%.

Detection of antibody levels (GMT)

We collected and separated serum from the immunized mice as described previously. The HI titers of their serum were detected by standard methods using each sub-type strain virus with 8 HA units, as published previously.¹¹ Serum neutralization antibody titers were tested by micro-neutralization (MN) assays.²³ Then, we calculated the geometric mean titers (GMTs) of specific antibodies.

Attenuation evaluation in ferrets

Since the ferret is a typical sensitive animal model for influenza virus, twelve ferrets at ten weeks old were purchased from Angora Ltd. (Jiangsu, China) at a male to female ratio of 1:1. Assessment of attenuation of virulence was implemented on them. The dosage was 500 µL per ferret with SI/06Vca.

The concentration gradients of virus were set as 10^5 TCID₅₀/mL, 10^6 TCID₅₀/mL and 10^7 TCID₅₀/mL, diluted with normal salt. It was administered by the n.i. pathway. Three days after immunization, we collected and separated their nasal mucosa tissue, bronchus, lungs and brain to detect the deposited SI/06Vca virus.

We ground each type of tissue and lysed them by sonication. The supernatant was separated. Then, we seeded this supernatant on Vero cells at 25°C, and the MOI was calculated.

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Peking Union Medical College (Permit Number: PUMC201411-0816731). All of the surgeries were performed under sodium pentobarbital anesthesia, and all efforts were undertaken to minimize suffering.

Analysis of efficacy against SI/06wt Challenge

Ferrets were intranasally challenged with 10 times the LD₅₀ of SI/06wt virus at 30 days post-vaccination). Mock-infected control mice received only 50 µl of PBS. The challenged ferrets were monitored and their symptoms, body weights and survival were recorded for another 14 days.

Statistics and data

All of the data are represented as the mean ± SD of three or more independent experiments. If the data were homogenous, analysis of variance, the Student-Newman-Keuls test and Pearson's correlation were used. If the data were not homogenous, the Kruskal-Wallis and the Games-Howell test, as well as a Spearman's correlation analysis, were used. All of the analyses were performed using SPSS software, version 19.0 (SPSS Inc, Chicago, IL, USA). Values less than 0.05 were considered to be statistically significant.

Disclosure of potential conflicts of interest

All authors declared that she has no conflict of interest.

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