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The stability and immunogenicity of inactivated MDCK cell-derived influenza H7N9 viruses

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ABSTRACT

In recent years, cell-based influenza vaccines have gained a great interest over the egg-based vaccines. Several inactivated H7N9 vaccines have been evaluated in clinical trials, including whole-virion vaccines, split vaccines and subunit vaccines. Recently, we developed a new suspension MDCK (sMDCK) cell line for influenza viruses production. However, the properties of purified antigen from sMDCK cells remain unclear. In this study, the stability of influenza H7N9 vaccine bulk derived from sMDCK cells was investigated, and the data were compared with the vaccine antigen derived from our characterized adhesion MDCK (aMDCK) cells in serum-free medium. The influenza H7N9 bulks derived from sMDCK and aMDCK cells were stored at 2–8 °C for different periods of time, and a number of parameters selected to monitor the H7N9 vaccine antigen stability were evaluated at each interval (1, 3 and 12 months). The monitored parameters included virus morphology, hemagglutinin (HA) activity, HA concentration, antigenicity, and immunogenicity. The sMDCK-derived H7N9 bulk showed similar morphology to that of the aMDCK-derived H7N9 bulk, and there were no obvious changes after the extended storage periods. Furthermore, the HA titer, HA concentration, and antigenicity of sMDCK-derived H7N9 bulk were stable after 28 months of storage. Finally, the results of hemagglutination inhibition and neutralization tests showed that sMDCK- and aMDCK-derived H7N9 vaccines had comparable immunogenicity. These results indicated that sMDCK-derived H7N9 bulk has good stability compared to that of aMDCK-derived H7N9 bulk. Thus, the newly developed suspension MDCK cell line shows a great alternative for manufacturing cell-based influenza vaccines.

1. Introduction

Vaccination is an effective method for preventing viral infection during influenza epidemics and pandemics; however, traditional egg-based production platform has limitations such as egg shortage during pandemics. The recent outbreak of H7N9 avian influenza in China is the fifth epidemic wave since March 2013, and raises a major concern for the world. Prior to August 7, 2017, there have been 1557 infected cases and 605 (39%) of them died. Adjuvanted H7N9 vaccines are shown to induce effective immune response against H7N9 virus [1]. In addition to vaccine efficacy, understanding vaccine stability is also critical factor for the design and management of immunization programs.

Stability evaluation of vaccine requires the appropriate physicochemical and biological assays to assess the changes in vaccine qualities, including antigen content and immune efficacy [2]. Hemagglutinin (HA) activity is a specific biological function of influenza virus and HA titer is a major functional indicator of influenza virus content during the manufacturing process. For the quantification of inactivated influenza vaccine antigens, the single radial immunodiffusion (SRID) assay is still the gold-standard method for measuring the content of HA antigen [3]. Vaccine efficacy depends primarily on the ability to elicit immune responses and the degree of similarity between the vaccine strain and circulating viruses [4]. Therefore, the following parameters including HA titer, HA concentration, virus morphology, antigenicity, and immunogenicity are required to monitor the stability of inactivated influenza vaccines.

In contrast to egg-based influenza vaccines, cell-based influenza vaccines have several advantages, including no dependency on egg supply, flexibility and scalability of the manufacturing process,

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better growth of most human influenza viruses, and antigenic similarity to circulating viruses [5]. We used characterized adhesion Madin-Darby canine kidney (aMDCK) cells for the production of inactivated whole-virion (IWV) H5N1 and H7N9 vaccines [6,7] in a cGMP bioproduction plant at National Health Research Institutes (NHRI), Taiwan. The influenza H5N1 and H7N9 IWV vaccine candidates using this manufacturing method have completed the human phase I and phase II trials, respectively [8,9]. However, the adherent MDCK-derived process requires complicated handling and labor-intensive procedure in large-scale operation. Several MDCK suspension cell lines have also been developed to overcome the problem of scalability in the adherent cell-based manufacturing process [10–13]. Recently, we developed a NHRI suspension MDCK (NHRI sMDCK) cells through serial adaptation to serum-free suspension culture without the use of microcarriers (patent pending, No. WO2017072744A1).

Several types of influenza H7N9 vaccines, including inactivated influenza vaccines, live-attenuated influenza vaccines, and recombinant-based virus-like particles, have been developed for clinical trials [14]. To enhance the preparedness of influenza outbreak, H7N9 bulk antigen and adjuvant were stockpiled separately [1]. But the long-term stability of IWV H7N9 bulk from NHRI sMDCK cells is remained unclear. Therefore, this study was aimed to evaluate the stability of IWV H7N9 bulk derived from newly developed sMDCK cells. This study also confirmed that the vaccine antigen derived from sMDCK cells has good characteristics throughout the stability program and that sMDCK cells is suitable for manufacturing influenza vaccines.

2. Materials and methods

2.1. Virus, cells and medium

The egg-derived influenza H7N9 reassortant vaccine virus (NIBRG-268) generated using reverse genetics was obtained from the UK National Institute of Biological Standard and Control (NIBSC). The NIBRG-268 virus was further adapted in aMDCK cells to increase its growth efficiency at NHRI. The antigenicity of aMDCK-adapted NIBRG-268 was confirmed using NIBRG-268 standard antisera (No. 13/180, NIBSC) to maintain similar antigenicity with NIBRG-268 virus [7]. The aMDCK cells (ATCC CCL-34) were purchased from the Food Industry Research and Development Institute, Hsinchu, Taiwan. Adhesion MDCK cells were cultured in OptiPro medium (GibcoBRL) supplemented with 4 mM glutamine (GibcoBRL). Suspension MDCK cells were cultured in BalanCD® Simple MDCK medium (Irvine Scientific) supplemented with 4 mM glutamine. For the viral growth in aMDCK and sMDCK cells, the culture media were supplemented with 2 μg/mL of TPCK-trypsin (Sigma).

2.2. Production of vaccine antigen in aMDCK or sMDCK cells

For the production of NIBRG-268 virus in aMDCK cells, the cells were sequentially expanded in T-flasks, roller bottles and a microcarrier-bioreactor. The aMDCK cells were grown on Cytodex1 microcarriers in 3.5 L disposable bioreactor (New Brunswick Scientific) for 5 days. When the cell density reached to approximately 1.6×10^6 cells/ml prior to infection, aMDCK-adapted NIBRG-268 virus was inoculated on the expanded aMDCK cells at a multiplicity of infection (MOI) of 0.0001 and harvested 3 days post-infection.

For the production of NIBRG-268 virus in sMDCK cells, sMDCK cells were grown in a 5 L bioreactor (Sartorius). When the cell concentration reached approximately 1.8×10^6 cells/mL, the aMDCK-adapted NIBRG-268 virus was inoculated at an MOI of 0.0001 and harvested 3 days post-infection.

2.3. Downstream purification processes

The H7N9 bulk, derived from sMDCK and aMDCK cells, was prepared as previously described [15]. Briefly, 1 L of harvested virus was clarified with a 0.65 μm Sartopure PP2 depth filter, inactivated with 0.01% formalin, and concentrated by tangential flow filtration (TFF) unit with a 300 kDa PESU membrane cassette (Sartorius). To remove host cell DNA and proteins from the harvest, the inactivated virus solution was separated using Capto Q and Capto core 700 anion exchange chromatography columns in an AKTA purifier 100 system (GE Healthcare). For the concentration and buffer exchange of virus fluid, the flow-through virus solution was diafiltered with phosphate buffered saline (PBS) using the TFF unit with a 100 kDa membrane cassette (Sartorius). Finally, 50 ml of bulk was obtained through a 0.22 μm sterile filtration prior to final storage.

2.4. Negative-stain electron microscopy (EM)

The negatively stained sample was prepared as follows: 4 μl purified bulk was adsorbed onto glow-discharged carbon coated grids (Electron Microscopy Sciences), washed once with a drop of ddH_2O, negatively stained with a drop of 2% uranyl acetate (UA) and air dried. The EM images of stained viral particles were captured using with a JEM-1400 at a magnification of 60,000 and with an accelerating voltage of 120 kV. The digital images were recorded using Gatan, Inc. Ultrascan 4000 4 k × 4 k Camera System (Model 895).

2.5. Hemagglutination assay (HA assay)

A more sensitive HA assay was used and modified according to the study of Kalbfuss *et al.* [16], with a mean confidence interval of +41%/−29%, to reduce the variation of the traditional discontinuous HA assay. Samples were serially diluted $1{:}2^{0.5}$ in round-bottomed 96-well microplates. Turkey red blood cells at a concentration of 0.25% were added to each well, and the plates were incubated for 2 h to 3 h at room temperature. The sedimentation of erythrocytes was evaluated by absorbance measurements at 700 nm, and HA titers were calculated as described by Kalbfuss *et al.* [16]. The HA titers were reported as hemagglutinin units (HAU)/50 μl.

2.6. SRID assay

The SRID assay was used to measure the amount of HA antigen as previously described [3]. The standard HA antigen (No. 14/250) and HA antiserum (No. 13/180) for H7N9 influenza virus, was purchased from the UK NIBSC. Briefly, the samples and HA standards were treated with 1% Zwittergent® 3–14 (Calbiochem) at room temperature for 30 min. Next, the serial dilutions of the antigen were loaded into wells on the 1% Seakem ME agarose gel (Lonza) mixed with anti-HA serum. Following incubation at room temperature overnight, the gel was dried and stained with Coomassie blue. Finally, the diameter of precipitant rings was measured and antigen content was calculated according to the linear curve of standard HA antigen.

2.7. Total protein and residual DNA measurement

The total protein concentration of harvests and purified bulks was detected by a modified Lowry assay kit (Thermo Fisher Scientific). Bovine serum albumin was used for the calibration

standards. Host cell DNA content of harvests and purified bulks was quantified using a Qubit dsDNA HS assay kit (Invitrogen). The detection range was 10 pg/μl–100 ng/μl. All samples were measured in triplicate.

2.8. Mice study

The mice study was approved by the Institutional Animal Care and Use Committee of NHRI (Protocol No: NHRI-IACUC-105129-A). Aluminum hydroxide ($Al(OH)_3$, Alhydrogel®) was purchased from Brenntag AG and given as a 300 μg dose. Six-week-old female BALB/c mice (n = 6 per group) were administered with $Al(OH)_3$-adjuvanted vaccines: 0.2 μg sMDCK- and aMDCK-derived H7N9 vaccine. All vaccines were administered twice at 2-week intervals (day 0 and day 14) by intramuscular injection into the quadriceps. On day 28, blood samples were collected into serum separator tubes (BD BioScience) and the serum was isolated by centrifuging at 3000 rpm for 10 min and was then stored at −20 °C.

2.9. Serological assays

The hemagglutination inhibition (HI) assay was used to assess the functional antibodies that inhibit agglutination of erythrocytes as previously described [17]. Briefly, mice sera were treated with a receptor-destroying enzyme (RDE) overnight and heat-inactivated for 30 min. Serial 2-fold dilutions of the sera were incubated with 4 HA units of inactivated H7N9 virus at room temperature for 15 min. Next, 0.5% Turkey red blood cells were added, and hemagglutination activity was observed after 40 min incubation. Serum HI titer was the reciprocal of the highest dilution of the serum inhibiting hemagglutination.

The neutralization (NT) assay was used to assess serum neutralizing antibodies that inhibit the infection of aMDCK cells with virus as previously described [17]. Briefly, serial 2-fold dilutions of mice sera were incubated with 100 $TCID_{50}$ of aMDCK-adapted NIBRG-268 H7N9 virus at 37 °C for 2 h. Serum-virus mixture was added to aMDCK cells and incubated at 35 °C for 96 h. Finally, neutralizing titers were evaluated by cytopathic effect and expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus.

3. Results

3.1. Design of stability program

sMDCK- and aMDCK-derived inactivated H7N9 bulks were stored at 2–8 °C for various periods of time prior to the initial time-point of the stability program (Fig. 1), and the original HA titer and HA protein concentration of each bulk are shown in Table 1. As an exception, the HA concentration of aMDCK-derived inactivated H7N9 bulks were measured only at the initial time-point of the stability program. This stability program lasted 12 months and the monitored parameters were analyzed at the 1st, 3rd and 12th month (Fig. 1).

3.2. Morphology of H7N9 bulks derived from different manufacturing platforms

EM analysis revealed that the morphology of the H7N9 viral particles derived from different manufacturing platforms displayed a spherical shape with a densely stained core (Fig. 2). During the course of the stability program, the morphology of the H7N9 viral particles derived from sMDCK and aMDCK cells was similar.

3.3. Stability of HA titer and HA concentration

After 12 months stored at 2–8 °C, the HA titer of sMDCK- and aMDCK-derived inactivated H7N9 bulks were comparable to the original HA titer (Fig. 3A). Similarly, the HA concentration of sMDCK- and aMDCK-derived inactivated H7N9 bulks showed no obvious reduction compared to the original HA concentration (Fig. 3B). These results suggested that the HA titer and HA concentration of inactivated H7N9 bulks derived from sMDCK and aMDCK cells were stable at 2–8 °C over the period of the stability study.

3.4. Stability of antigenicity and immunogenicity

To investigate the efficacy of bulk antigens over the period of the stability program, the antigenicity and immunogenicity of the H7N9 bulks derived from sMDCK and aMDCK cells were analyzed. The reactivity of reference antiserum against A/Anhui/1/2013 virus with sMDCK- and aMDCK-derived H7N9 bulks was

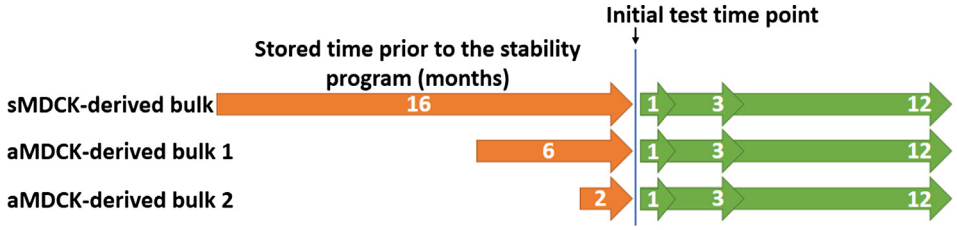

Fig. 1. Test schedule for the stability program of H7N9 bulks derived from different manufacturing platforms. sMDCK- and aMDCK-derived H7N9 bulks were stored at 2–8 °C for different periods of time, and a number of parameters were measured to monitor the H7N9 vaccine antigen stability at different periods (1st, 3rd and 12th month). These monitoring parameters included virus morphology, HA titer, HA concentration, antigenicity, and immunogenicity.

Table 1
Original HA titer and HA concentration of different tested bulks.

	Bulk				Mouse dose	
	HA titer (HAU/50 μl)	HA protein (μg/ml)	Protein (μg/ml)	DNA (ng/ml)	Protein (μg)	DNA (ng)
sMDCK-derived bulk	18,428	122.7	351.2	263	0.57	0.43
aMDCK-derived bulk 1	3537	34.5[a]	106.7	2	0.62	0.30
aMDCK-derived bulk 2	1522	15.0[a]	45.6	45	0.61	0.60

Note: Protein and DNA levels of different tested bulks were measured by the Lowry assay. Protein and DNA concentrations per mouse dosage were based on 0.2 μg HA protein.
[a] HA concentration was measured at the initial time point of the stability study.

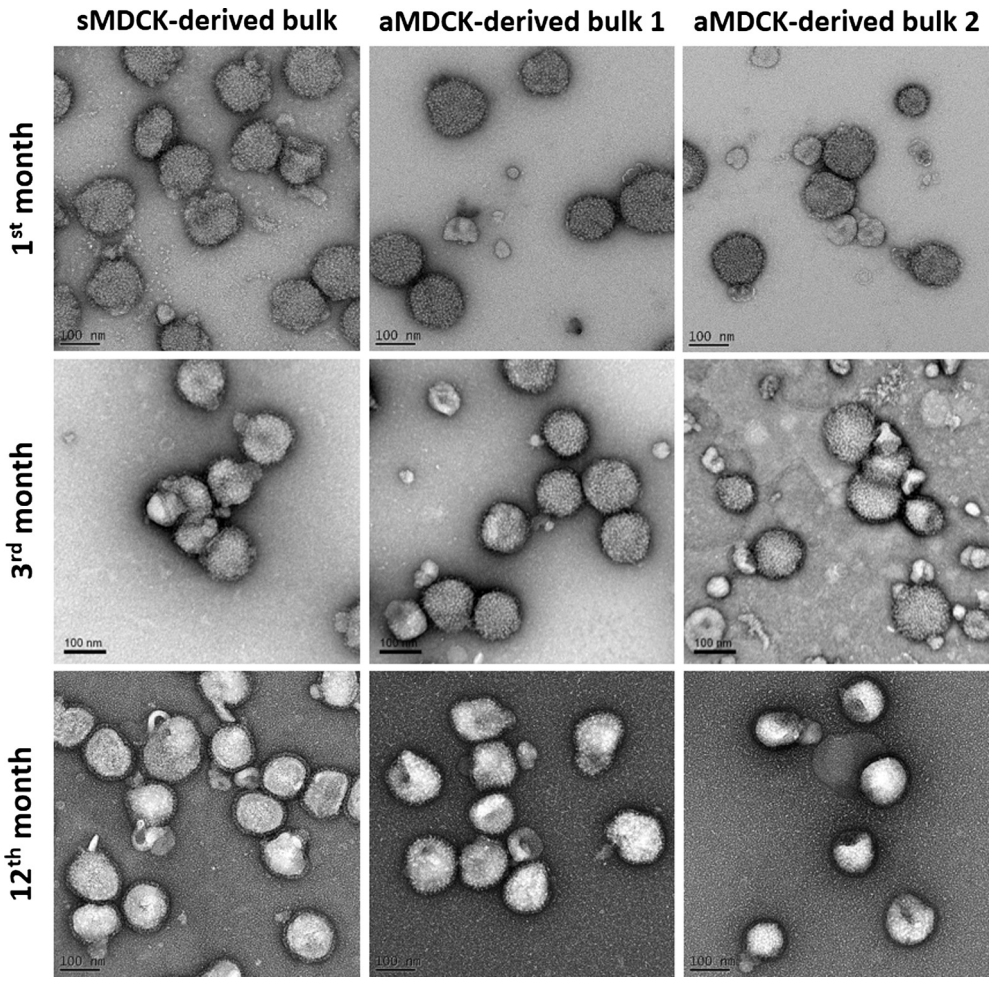

Fig. 2. EM images of H7N9 bulks from different platforms. sMDCK- and aMDCK-derived H7N9 bulks were stored at 2–8 °C, and the morphology of the H7N9 viral particles was analyzed at the 1st, 3rd and 12th month of the stability program. Viral particles were negatively stained with 2% uranyl acetate and the images were captured using electron microscopy.

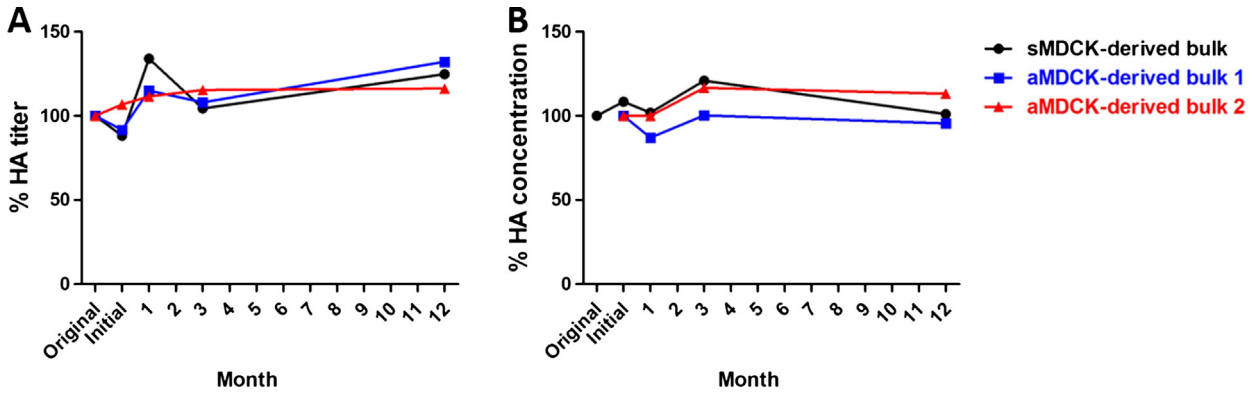

Fig. 3. HA titer and HA concentration of H7N9 bulks from different platforms during the stability study. sMDCK- and aMDCK-derived H7N9 bulks were stored at 2–8 °C, and their HA titer (A) and HA concentration (B) were measured at the indicated time points by HA and SRID assays, respectively. The relative HA titer and HA concentration were expressed as a percentage relative to the original value or the value detected at the initial time point of the stability program.

studied. The HI titers of sMDCK- and aMDCK-derived H7N9 viruses ranged from 640 to 1280 over the period of the stability program (Table 2), indicating that the antigenicity of sMDCK-derived H7N9 virus was similar to that of aMDCK-derived H7N9 virus and that the antigenicity of sMDCK- and aMDCK-derived H7N9 viruses was stable after 12 months stored at 2–8 °C.

To explore the immunogenicity of the H7N9 bulks derived from sMDCK and aMDCK cells, the bulks were mixed with $Al(OH)_3$ adjuvant and 6-week-old BALB/c mice were immunized intramuscularly with 2 doses of vaccine antigen at a 2-week interval. Table 1 showed total protein and DNA levels in different tested bulks, and the dosage content for mice study. Total protein and

Table 2
Antigenic analysis of sMDCK- and aMDCK-derived H7N9 bulks during the stability study.

	Test time point of the stability program		
	1st month	3rd month	12th month
sMDCK-derived bulk	1280	1280	1280
aMDCK-derived bulk 1	640	1280	1280
aMDCK-derived bulk 2	1280	1280	1280

Note: sMDCK- and aMDCK-derived H7N9 bulks were stored at 2–8 °C, and their antigenicity were measured at the indicated time points by hemagglutination inhibition (HI) assay using reference antiserum.

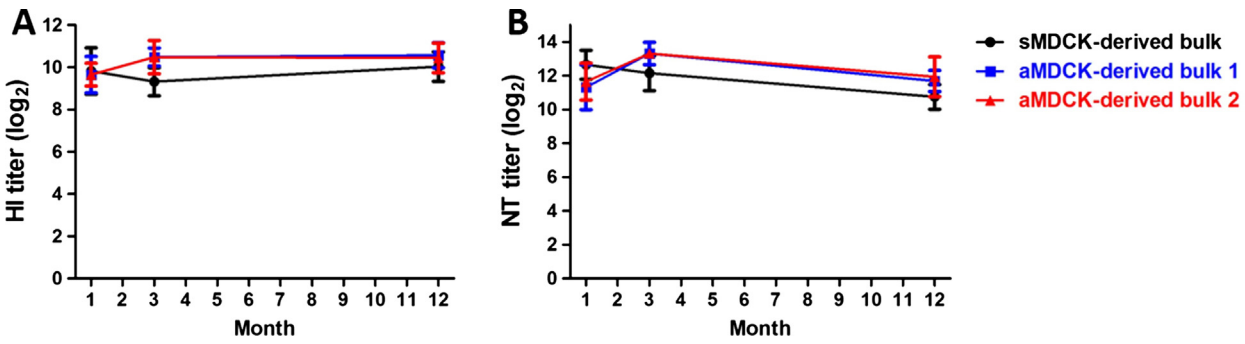

Fig. 4. Immunogenic analysis of various H7N9 bulks during the stability study. sMDCK- and aMDCK-derived H7N9 bulks were stored at 2–8 °C. 0.2 μg of the HA antigen dose of MDCK-derived H7N9 bulks from the indicated time points were mixed with $Al(OH)_3$ adjuvant, and administered in BALB/c mice (n = 6 per group) intramuscularly at day 0 and day 14. The immunogenicity of various bulks was confirmed by HI and NT assays, using the serum collected at day 28. Error bars represent the 95% confidence interval.

residual DNA in each dose did not more than 0.62 μg and 0.6 ng, respectively. The level of anti-H7N9 antibody at 2 weeks after the second immunization was measured by HI and NT assays, and aMDCK-derived bulk 2 and NIBRG-268 virus were used as antigens in the HI assay and infectious virus in the NT assay, respectively. At the 1st month of the stability program, the HI titers of sMDCK- and aMDCK-derived H7N9 vaccines ranged from 806 to 905 against aMDCK-derived bulk 2 (Fig. 4A), and the NT titers ranged from 2560 to 6451 against NIBRG-268 virus (Fig. 4B). The change in the level of anti-H7N9 antibody was less than 4-fold and similar results were observed at the 3rd and 12th month of the stability program (Fig. 4). These results showed that the sMDCK-derived bulk retained an antigenicity similar to that of aMDCK-derived bulks, and the antigenicities from both sMDCK and aMDCK cells were stable after 12 months stored at 2–8 °C.

4. Discussion

Our results showed that the antigenicity and immunogenicity of sMDCK-derived NIBRG-268 H7N9 bulk were maintained stable for over 28 months (Fig. 3B). This data demonstrated that NIBRG-268 strain derived from the first-wave A/Anhui/1/2013 H7N9 virus is suitable for the manufacturing process of inactivated whole-virion vaccines using sMDCK cells. However, the antigenicity of the highly pathogenic H7N9 viruses in the fifth epidemic wave is different from that of the first-wave H7N9 virus [18]. Several amino acid differences were found in the hemagglutinin head region of highly pathogenic H7N9 viruses [19]. The experience in influenza A (H1N1) pdm09 showed that amino acid substitution in vaccine strain could change conformational stability, suggesting that the stability of the bulk antigen is strain dependent [20]. Recently, several vaccine strains for the fifth-wave H7N9 virus have been developed for pandemic preparedness [21]. Further research is required to understand the vaccine stockpile durability of MDCK-derived the fifth-wave H7N9 vaccine strain.

In conclusion, this study evaluated the long-term stability of MDCK-derived H7N9 bulks stored at 2–8 °C. The concentration of HA antigen is a main characteristic in the stability testing of vaccine bulk. The antigens of sMDCK- and aMDCK-derived H7N9 bulks were stable for over one year, which should be sufficient to cover the entire shelf-life. Adhesion MDCK cells have been characterized for the production of influenza vaccine, but are still relatively inconvenient during scale-up of manufacturing processes. The HA titer and content of sMDCK-derived H7N9 bulk were stable for at least 28 months after manufacture, and vaccine efficacy of sMDCK-derived H7N9 bulk was similar to that of aMDCK-derived H7N9 bulk. Therefore, our newly developed suspension MDCK cell line solved the issues of aMDCK cells grown in large-scale bioreactors and also showed a good stability of viral bulk over a long period of time. The sMDCK cell line can be considered another potential host cell for manufacturing cell-based influenza vaccines.

Conflict of interest

None of the authors has any conflicts of interest.

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