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Rapid production of a H_9N_2 influenza vaccine from MDCK cells for protecting chicken against influenza virus infection

Zhenghua Ren · Zhongzheng Lu · Lei Wang · Zeren Huo · Jianhua Cui · Tingting Zheng · Qing Dai · Cuiling Chen · Mengying Qin · Meihua Chen · Rirong Yang

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Abstract H_9N_2 subtype avian influenza viruses are widespread in domestic poultry, and vaccination remains the most effective way to protect the chicken population from avian influenza pandemics. Currently, egg-based H_9N_2 influenza vaccine production has several disadvantages and mammalian MDCK cells are being investigated as candidates for influenza vaccine production. However, little research has been conducted on low pathogenic avian influenza viruses (LPAIV) such as H_9N_2 replicating in mammalian cells using microcarrier beads in a bioreactor. In this study, we present a systematic analysis of a safe H_9N_2 influenza vaccine derived from MDCK cells for protecting chickens against influenza virus infection. In 2008, we isolated two novel H_9N_2 influenza viruses from chickens raised in southern China, and these H_9N_2 viruses were adapted to MDCK cells. The H_9N_2 virus was produced in MDCK cells in a scalable bioreactor, purified, inactivated, and investigated for use as a vaccine. The MDCK-derived H_9N_2 vaccine was able to induce high titers of neutralizing antibodies in chickens of different ages. Histopathological examination, direct immunofluorescence, HI assay, $CD4^+/CD8^+$ ratio test, and cytokine evaluation indicated that the MDCK-derived H_9N_2 vaccine evoked a rapid and effective immune response to protect chickens from influenza infection. High titers of H_9N_2-specific antibodies were maintained in chickens for 5 months, and the MDCK-derived H_9N_2 vaccine had no effects on chicken growth. The use of MDCK cells in bioreactors for LPAIV vaccine production is an attractive option to prevent outbreaks of LPAIV in poultry.

Keywords H_9N_2 · MDCK · Vaccine · Bioreactor

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Z. Ren · M. Chen
National Engineering Research Center of South China Sea Marine Biotechnology Co., Ltd, Haizhu Technology building, 2429 East Xingang Road, Guangzhou 510330, People's Republic of China

Z. Ren · L. Wang · T. Zheng · Q. Dai · C. Chen · M. Qin
Department of Biochemistry, School of Life Sciences, Sun Yat-sen (Zhongshan) University, 135 West Xingang Road, Guangzhou 510275, People's Republic of China

Z. Lu
Shenzhen, South China Sea tide Biotechnology Co., Ltd, Minning Garden Office Building Room 905, Cai Tian Road, Futian District, Shenzhen, Guangdong 518000, People's Republic of China

Z. Huo
Sun Yat-Sen Memorial Hospital, Sun Yat-Sen (Zhongshan) University, 107 West Yanjiang Road, Guangzhou 510120, People's Republic of China

J. Cui
Thoracic Surgery of China-Japan Union Hospital, Jilin University, Changchun 130033, People's Republic of China

R. Yang (✉)
Department of Immunology, School of Preclinical Medicine, Biological Targeting Diagnosis and Therapy Research Center, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, People's Republic of China
e-mail: 520yrr@163.com

Introduction

Influenza A virus is a widespread pathogen and can cause infections both in birds and human beings (Lamb and Takeda 2001), resulting in human suffering and agricultural economic burden. Based on genetic features and/or severity of the disease in poultry, influenza viruses are classified as either highly pathogenic avian influenza virus (HPAIV) strains or low pathogenic avian influenza virus (LPAIV) strains that cause asymptomatic or moderate infections (Lee and Song 2013; Tombari et al. 2011). The H_9N_2 genotype is a stereotypical LPAIV and, since 1994, H_9N_2 influenza viruses have

become the most prevalent subtype in domestic poultry in Asia (Guo et al. 2000; Lee and Song 2013; Liu et al. 2003; Nili and Asasi 2003; Wu et al. 2008). This has resulted in reduced egg production and increased mortality among poultry. Furthermore, avian-to-human transmission of H_9N_2 virus has been reported in China, indicating that H_9N_2 viruses pose potential threats to public health (Butt et al. 2005; Cameron et al. 2000; Lin et al. 2000).

Vaccination is the prevalent strategy to prevent outbreaks of avian influenza (Genzel and Reichl 2009). In the past few years, seasonal influenza vaccines have been manufactured using embryonated chicken eggs. However, this approach has many disadvantages. Firstly, the egg-based vaccine relies on a supply of specific pathogen-free (SPF) eggs, which is limited and available only with advanced planning (Doroshenko and Halperin 2009; Tree et al. 2001). Secondly, egg-based influenza virus cultivation can lead to the selection of viruses harboring variants in the hemagglutinin protein (Govorkova et al. 1996). Thirdly, influenza vaccine production in eggs is not adequate for the immediate, rapid, large-scale vaccine production which is required in the event of an influenza pandemic, and therefore, this method is only able to accommodate a small percentage of the infected population (Doroshenko and Halperin 2009; Hu et al. 2011). Moreover, the emergence of new viral strains responsible for global pandemics requires a switch in vaccine production from the seasonal to the pandemic vaccine. However, the pandemic vaccine manufacturing processes are currently a bottleneck and unable to meet the global vaccination demand (Bock et al. 2011; Hu et al. 2011; Liu et al. 2012).

Propagation of influenza virus in mammalian cells is an alternative to the egg-based process for influenza vaccine production. Two continuous cell lines, Madin-Darby canine kidney (MDCK) cells and African green monkey kidney (Vero) cells, have been investigated for their use in HPAIV propagation (Genzel et al. 2004; Govorkova et al. 1999; Hu et al. 2008; Tree et al. 2001; Youil et al. 2004). For the differences among influenza virus subtypes, the sensitivity of continuous cell lines to influenza virus is different and the cultivation of LPAIV in continuous cell lines has not been established yet. MDCK cells are fully characterized, and a defined culture medium formulation gives consistent results for MDCK cell growth and virus propagation (Hu et al. 2011; Tree et al. 2001). Due to the consistently high viral titers produced by MDCK cells (Hu et al. 2011; Tree et al. 2001), production of MDCK-derived vaccine offers advantages over the egg-based method. Nevertheless, one limitation of MDCK cells is that these cells require surface adhesion to proliferate, and the cultivation of MDCK cells on a flat surface is not easy to scale up for industrial influenza virus production. The suspension MDCK is available (Doroshenko and Halperin 2009). Because of intellectual property rights and the proprietary technologies, there is little information available on the suspension MDCK culturing system. An alternative solution for the cell-based production of influenza vaccine is the introduction of microcarrier beads to a bioreactor. This method provides sufficient surface area so that a high yield of virus can be obtained (Genzel et al. 2006; Hu et al. 2008).

In recent years, tremendous progress has been made in improving the cultivation of HPAIVs in MDCK cells using microcarrier beads in bioreactors (Bock et al. 2011; Genzel et al. 2004; Genzel et al. 2006; Hu et al. 2011; Hu et al. 2008; Liu et al. 2012; Tree et al. 2001), and some investigations on the immunogenicity and reactogenicity of the MDCK-derived HPAIV vaccines have been reported (Doroshenko and Halperin 2009; Palache et al. 1997). Nevertheless, differences among the influenza virus subtypes require modifications to the microcarrier-based MDCK cell culture conditions, including the medium formulation, agitation speed, pH, dissolved oxygen, and temperature (Bock et al. 2011; Genzel et al. 2004; Genzel et al. 2006; Hu et al. 2011; Hu et al. 2008; Liu et al. 2012; Tree et al. 2001). When a novel influenza virus subtype is adapted to proliferate in continuous cell lines, the immunogenicity and safety of the corresponding inactivated influenza vaccine needs to be further analyzed through the use of a relevant animal model.

Three HPAIV subtypes (H_1N_1, H_3N_2, and H_5N_1) have been adapted for replication in MDCK cells (Genzel and Reichl 2009). However, little research has been conducted on the production of LPAIV, such as the H_9N_2 virus, using mammalian host cells in a bioreactor. In our study, we isolated two novel H_9N_2 subtype influenza viruses and adapted them to MDCK cells. The adapted H_9N_2 virus was then cultivated in a microcarrier-based MDCK cell culture system to rapidly produce the inactivated H_9N_2 vaccine. Antigenicity and immunogenicity analyses were performed in chickens, and the MDCK-derived H_9N_2 vaccine consistently elicited an immune response to protect chicken from influenza virus infection.

Materials and methods

Biological materials

H_9N_2 influenza A virus strains A/chicken/Guangdong/GD01/2008 (strain number GD01) and A/chicken/Guangdong/GD03/2008 (strain number GD03) were deposited in the Zhongda Biosafety Level-3 Laboratory of Guangdong Dahuanong Animal Health Products Co., Ltd. (No. CNAS BL0023) and supplied by the School of Life Sciences at Sun Yat-Sen University. The H_9N_2 influenza A virus strains GD01 and GD03 were publicly available by contacting the author Zhenghua Ren (Tel.: +86 020 39332956, E-mail: renzhh@mail.sysu.edu.cn) or the corresponding author

Rirong Yang. MDCK cells (ATCC catalog no. CRL-2935) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % heat-inactivated fetal bovine serum (FBS; Gibco) (complete medium). The serum-free medium was just DMEM. SPF chickens and chicken embryos were purchased from Guangdong Dahuanong Animal Health Products Co., Ltd.

RNA isolation, reverse transcription, and PCR

Total RNA was isolated from influenza virus samples using TRIzol LS Reagent (Life Technologies) and subjected to reverse transcription (Toyobo). PCR was carried out using PrimeSTAR HS DNA polymerase (Takara), and primers used for amplification of hemagglutinin (HA), neuraminidase (NA), and matrix protein 1 (M1) genes are listed in Table S1. The identity of PCR amplicons was then verified by direct sequencing. The HA, NA, and M1 gene sequences of H_9N_2 influenza A virus strains GD01 and GD03 were submitted to GenBank, and the corresponding protein sequences were annotated by using Influenza Virus Sequence Annotation Tool in Influenza Virus Resource (Bao et al. 2008).

Analyses of H_9N_2 influenza virus HA, NA, and M1 sequences

All referenced H_9N_2 sequences were obtained from the Influenza Virus Database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) (Bao et al. 2008). DNA or amino acid sequences (GenBank accession numbers are listed in Tables 1 and 2) were analyzed using the BLAST tool at the NCBI Web site, and multiple sequence alignment was performed using ClustalX 1.83 software (http://www.clustal.org/). The phylogenetic and molecular evolutionary analyses were carried out with MEGA4.1 software using the neighbor-joining method (Tamura et al. 2007).

Hemagglutination assay

Following the standard technique described by the World Health Organization manual (World Health Organization 2002), titration of influenza virus by hemagglutination assay was carried out in V-bottom 96-well plates. Serial twofold dilutions of virus samples were mixed with an equal volume of a 0.5 % cell suspension of chicken erythrocytes and incubated at room temperature for 30 min. The highest dilution of virus showing complete hemagglutination was taken as the HA titration end point and was expressed as $\log_2$ HA units per test volume (100 μL).

Cell and virus cultivation in the bioreactor

Cytodex 1 microcarrier beads were pretreated according to the manufacturer's instructions (GE Healthcare). After washing with PBS, twelve grams of Cytodex 1 microcarriers were added to a bioreactor with 2 L of complete medium. Approximately 10.3×10^8 MDCK cells in 1 L of complete medium were seeded, and the cultivation was performed in a 5-L bioreactor with 3 L of working volume. The agitation speed, pH, dissolved oxygen, and temperature were maintained at 60 rpm, 7.0, 50 % air saturation, and 37 °C, respectively. During the MDCK cell culture, MDCK cells were sampled daily for photomicrography (×100) and the cell density was measured by cell counting. After the complete medium was exchanged for serum-free medium, GD01 H_9N_2 influenza virus was added into the bioreactor for infection at a multiplicity of infection (MOI) of 0.05. For virus cultivation, the agitation speed, pH, dissolved oxygen, and temperature were maintained at 100 rpm, 7.2, 50 %, and 33 °C, respectively. MDCK cells were subsequently sampled daily, and the HA titer was determined using the hemagglutination assay. To detect glucose consumption, glucose concentration was measured using a GlucCell glucose monitoring system (CESCO Bioengineering) and maintained at 330 mg/dl.

Electron microscopy

The cultivation medium during influenza virus production in the bioreactor was collected. Following centrifugation, the supernatant was concentrated and purified using sucrose density gradient zonal centrifugation. The fraction between 40 and 50 % sucrose density was collected, and the sucrose was removed using ultracentrifugation. Purified virus particles were examined by transmission electron microscopy (JEOL JEM1400 , Japan).

HI assay

Following the standard procedure outlined in the WHO manual (World Health Organization 2002), serum H_9N_2 specific hemagglutination inhibition (HI) antibody titers were measured using the HI assay. The sera were treated with receptor-destroying enzyme. Serial twofold dilutions of sera were mixed with four HA units of the H_9N_2 influenza virus antigen at room temperature for 30 min. An equal volume of a 0.5 % cell suspension of chicken erythrocytes was added and incubated at room temperature for 30 min. The HI titer was expressed as the reciprocal of the highest dilution of antiserum that completely inhibited hemagglutination.

Table 1 GenBank accession numbers for H_9N_2 influenza A virus strains included in phylogenetic analysis

Strain	HA gene	NA gene	M1 gene
A/chicken/Guangdong/GD01/2008	KP100807	KP100809	KP100811
A/chicken/Guangdong/GD03/2008	KP100808	KP100810	KP100812
A/chicken/Guangdong/V/2008	JQ639786.1	JQ639788.1	JQ639789.1
A/chicken/Guangdong/TS/2004	JQ639778.1	JQ639780.1	–
A/Duck/HongKong/Y280/97	AF156376.1	AF156394.1	AF156461
A/Chicken/Beijing/1/94	AF156380.1	AF156398.1	–
A/swine/HongKong/10/98	AF222811	AF222813	–
A/Quail/HongKong/G1/97	AF156378	AF156396.1	AF156463
A/HongKong/1073/99	AJ404626	AJ404629	–
A/duck/HongKong/Y439/1997	KF188265	KF188267	AF156462
A/chicken/Hebei/B1/2001	EU573938	EU346934	EU532029.1
A/chicken/Shandong/B1/1998	EU573939	EU935061	EU532056.1
A/chicken/Beijing/L1/2005	EU573940	EU346935	EU532030.1
A/chicken/Henan/L1/2002	–	EU346936	EU532031.1
A/chicken/Jiangsu/L1/2004	EU939150	EU346937	EU532032.1
A/chicken/Shandong/B4/2007	EU939144	EU346938	EU532033.1
A/chicken/Zibo/B1/2008	EU939147	EU935062	EU835746.1
A/chicken/Tianjin/B1/2004	EU939151	EU346939	EU532034.1
A/chicken/Jiangsu/L2/2005	EU939152	EU346940	EU532035.1
A/chicken/Hebei/L1/2006	EU573941	EU346941	EU532036.1
A/chicken/Shandong/B2/2007	EU935071	EU340028	EU414522.1
A/chicken/Shandong/L1/2007	EU939145	EU939162	EU835747.1
A/chicken/Henan/L1/2008	EU939148	EU935063	EU835748.1
A/chicken/Henan/L3/2008	FJ547479	EU935064	EU835750.1
A/chicken/Henan/L2/2008	EU939149	FJ492965	EU835749.1
A/chicken/Zibo/L2/2008	EU939146	FJ492964	EU882860.1
A/chicken/Henan/43/02	DQ064369	DQ064423	DQ064396.1
A/chicken/Guangdong/4/00	DQ064358	DQ064412	DQ064385.1
A/chicken/China/Guangxi17/2000	DQ485224	DQ485226	DQ485227.1
A/chicken/China/Guangxi1/2000	DQ485208	DQ485210	DQ485211.1
A/chicken/Shandong/B3/2007	–	–	EU532028.1

Viral challenge assay in chicken

Experiments involving animals were approved by the Institutional Animal Care and Use Committee at Sun Yat-Sen University. All animal experiments using chickens treated with H_9N_2 virus were carried out in a biosafety level (BSL) 3 containment laboratory, the School of Life Sciences, Sun Yat-Sen University.

Purified GD01 H_9N_2 virus was derived from MDCK cells and inactivated with formalin and emulsified with liquid paraffin aluminum stearate suspension. The HA antigen concentration of the MDCK-derived H_9N_2 vaccine was 4.3 ng/0.1 mL measuring by H9N2 HA ELISA Pair Set (Sino Biological Inc.).

Each treatment group comprised five SPF chickens. Different doses of MDCK-derived H_9N_2 influenza vaccine (0, 50, 100, 200, and 500 μL) were injected subcutaneously into the chicken necks. Twenty-eight days later, chicken blood was collected and the H_9N_2-specific HI antibody titer was

Table 2 GenBank accession numbers for H_9N_2 influenza A virus strains used for the analysis of critical amino acid residues in the HA and NA proteins

Strain	HA protein	NA protein
A/chicken/Guangdong/V/2008	AFC98347.1	AFC98349.1
A/chicken/Guangdong/TS/2004	AFC98358.1	AFC98360.1
A/Duck/Hong Kong/Y280/97	AAF00704.1	AAD49004.1
A/Chicken/Beijing/1/94	AAF00708.1	AAD49008.1
A/Swine/Hong Kong/10/98	AAL14081.1	AAL14083.1
A/Quail/Hong Kong/G1/97	AAF00706.1	AAD49006.1
A/Hong Kong/1073/99	CAB95856.1	NP_859038.1
A/Duck/Hong Kong/Y439/97	AAF00705.1	AAD49005.1

determined by performing HI assays. Each chicken was then intravenously inoculated through the wing vein with 200 μL of GD03 H_9N_2 influenza virus (HA titer=9.5 $\log_2$ HA units/100 μL, 10^7 EID_{50}/100 μL, 1:10 dilution for use) (Li et al. 2012). Five days later, a cloacal sample from each chicken was collected with a cotton swab. To determine whether the vaccinated chickens were infected by the GD03 H_9N_2 virus, the swab was washed with 1 mL PBS and the rinse solution was collected. After filter sterilization, five sets of 100-μL rinse solution were injected into the allantoic cavities of five 9-day-embryonated chicken eggs, respectively. Following 5 days of incubation in eggs, the GD03 H_9N_2 virus titer of the allantoic fluid was determined by using the HA titer assay.

Vaccinations of 21-day-old chickens using either 200 μL of MDCK-derived vaccine or egg-derived vaccine were conducted as described above. Nonvaccinated and uninfected chickens were used as untreated controls. Blood samples were collected from these chickens on days 14, 21, and 28 following vaccination for flow cytometry assays. On the 28th day, these chickens were infected by GD03 H_9N_2 virus. The infected chickens without vaccination treatment were used as challenge controls. Five days later, lung tissues were dissected and examined using histopathological analysis, direct immunofluorescence assay, and quantitative real-time PCR, and the serum was obtained for cytokine evaluation.

To evaluate the duration of immunity after MDCK-derived vaccine immunization, 200 21-day-old SPF chickens were divided into four groups of 50 chickens. One group, which was nonvaccinated and uninfected but treated with PBS, was used as a negative control (NC). Other groups were vaccinated with 200 μL of MDCK-derived vaccine and housed for various lengths of time (7, 14, 21, 28, 35, 60, 90, 120, 150, and 180 days). At each time point, blood from five chickens was collected for HI assays. These chickens were then challenged with the GD03 H_9N_2 virus for 5 days, and infection of the vaccinated chickens was determined as described above. The body weight of the chickens in each group was monitored for 28 days.

Histopathological analysis and direct immunofluorescence

Lung tissue was fixed in 4 % paraformaldehyde at 4 °C overnight, dehydrated in a graduated ethanol series, and embedded in paraffin. The embedded tissues were cut into 14-μm sections. After deparaffinization and rehydration, tissue sections were treated with hematoxylin and eosin stain. For direct immunofluorescence, rehydrated tissue sections were pretreated with 10 % FBS blocking buffer at room temperature for 30 min, then incubated at 37 °C for 1 h with a primary antibody (fluorescein isothiocyanate (FITC)-conjugated anti-influenza A nucleoprotein monoclonal antibody, ViroStat). After washing steps with PBS, samples were stained with 0.2 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 3 min. Images were obtained with a fluorescence microscope (×100, Zeiss AxioVision 4 microscope, Germany).

Quantitative real-time PCR

Total RNA was isolated from lung tissue and treated with DNase I (Ambion), and then subjected to reverse transcription following the instructions of the TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara). The expression of 12 genes was analyzed, and the specific primer sequences are listed in Table S1. Real-time PCR was performed using the LightCycler480 (Roche), and reaction conditions for amplification were as follows: 40 cycles of a two-step PCR (95 °C for 15 s, 60 °C for 20 s) after an initial denaturation (95 °C for 30 s) step. *GAPDH* was used as an internal control for gene expression quantification. All reactions were run in triplicate, and relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Flow cytometry assay

Using lymphocyte separation medium (Sigma-Aldrich), peripheral blood mononuclear cells were separated from whole blood. Cells were then treated with 0.8 % ammonium chloride at room temperature for 10 min. After washing three times with PBS, the cells were incubated with mouse anti-chicken FITC-conjugated CD4 antibody or mouse anti-chicken FITC-conjugated CD8 antibody (ABD Serotec) in the dark at room temperature for 1 h, then were evaluated by the Flow Cytometry System (BD FACSCalibur).

ELISA

Chickens were vaccinated for 28 days as described above, and then challenged with GD03 H_9N_2 virus for 5 days. Peripheral blood was collected, and serum was separated using centrifugation. The concentration of IFN-γ and IL-18 in serum samples was measured using chicken-specific ELISA kits (Rapidbio) according to the manufacturer's protocol.

Statistical analyses

All experiments were repeated at least three times with three replicates per sample, and results are expressed as mean±standard deviation (SD), unless otherwise stated. GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA) was employed for statistical analysis. Comparisons between two groups were conducted using two-sided Student's *t* tests. Multiple comparisons among groups were performed by one-way ANOVA, followed by Tukey's honest significant difference (HSD) test. A p value <0.05 was considered statistically significant.

Results

Characterization of two novel influenza H_9N_2 viruses

In 2008, two influenza virus strains were isolated in the Guangdong province of China and initially identified as H9 subtype via the HI assay. Moreover, three genes encoded by these two influenza virus strains (HA, NA, and M1 genes) were sequenced and analyzed using BLAST. Based on multiple sequence alignment of both HA and NA gene sequences, these two isolates were identified as H_9N_2 subtypes and named A/chicken/Guangdong/GD01/2008 and A/chicken/Guangdong/GD03/2008. Phylogenetic trees constructed from alignments of H_9N_2 M1, HA, and NA gene sequences revealed that the two isolates belong to the Y280-like lineage (Figs. 1a and S1) and showed high similarity to the A/chicken/Guangdong/V/2008.

The hemagglutinin cleavage motif sequence of the two H_9N_2 isolates was identified as RSSRGL, and the key amino acid residues in the receptor binding site motif are conserved (Table 3). Noticeably, both HAs of the two H_9N_2 isolates contained a 234Q residue (H3 number 226) that has been demonstrated to bind to 2,3-linked sialic acid in avian receptors (Matrosovich et al. 1997). Seven potential glycosylation sites (N-X-T/S, where X can be any amino acid except proline) in the HA peptide sequence were found in the two H_9N_2 isolates. These sites are identical except that the HA protein of GD01 contains an alanine (A) at position 494.

Although the deletion of three amino acids (62–64) in the stalk of the NAs was not detected, significant changes were observed in both the hemadsorbing site and drug binding pocket of NA in the two isolates (Table 4). Like the A/chicken/Guangdong/V/2008 strain (Table 4), the chief differences in the hemadsorbing site are at position 367 (lysine to glutamate) and at position 403 (tryptophan to serine). In the drug binding pocket motif, position 432 was mutated from glutamine to lysine (Table 4).

Adaptation of H_9N_2 influenza virus to MDCK vells

H_9N_2 virus GD01 was adapted to proliferate in MDCK cells. MDCK cells were grown to confluency in six-well plates at 37 °C (about 3 days); then, the complete medium was exchanged for serum-free medium, and GD01 (MOI=0.05) was added for incubation at 33 °C. The resulting cytopathic effect was pronounced (Fig. 1b). As an indicator for cell metabolism, daily glucose uptake was monitored (Fig. 3c). Glucose consumption increased, peaked at the third day, and then decreased (Fig. 3c). The culture medium was collected on the third day after virus inoculation. After purification of virus particles from the culture medium, the typical structure of H_9N_2 viruses was identified morphologically by electron microscopy (Figs. 1c and S2a).

The growth of MDCK cells on microcarriers in a bioreactor

Twelve grams of Cytodex 1 microcarrier beads and 3 L of culture medium were added into a 5-L bioreactor, and MDCK cells were seeded using a ratio of approximately 20 cells per microcarrier. MDCK cells attached to the surface of the microcarriers and grew rapidly in the following 72 h (Fig. 2a, 24–72 h). On the third day after seeding, the cell density reached approximately 3.9×10^6 cells/mL. However, there was no significant difference between the cell density at

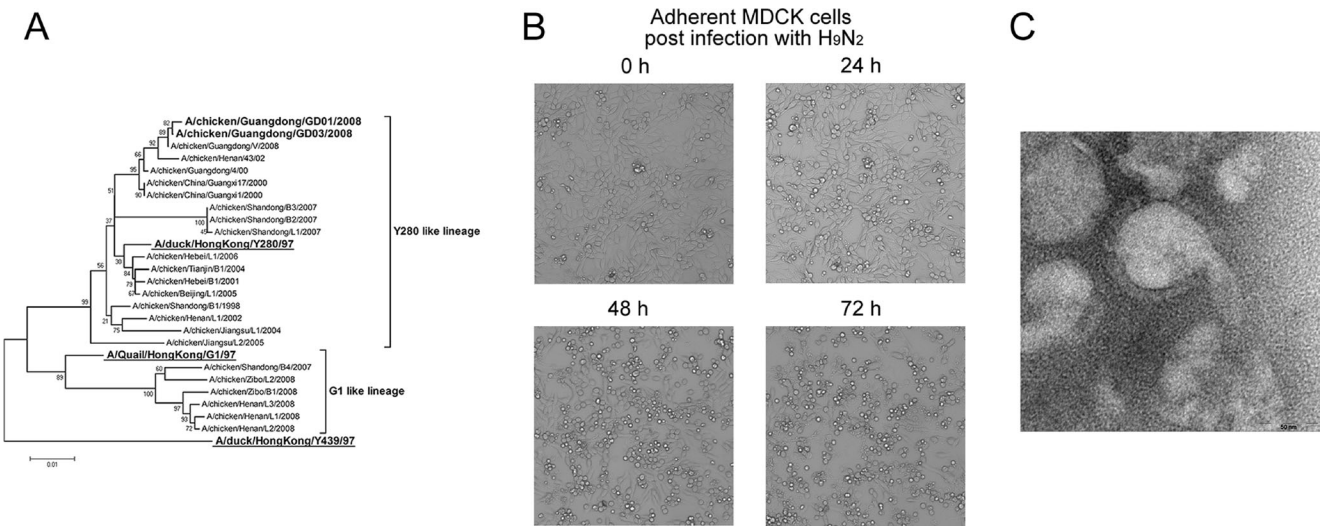

Fig. 1 Adaptation of the novel H_9N_2 isolate to MDCK cells. **a** Phylogenetic relationship of M1 genes in H_9N_2 influenza viruses. A neighbor-joining tree of H_9N_2 viruses was constructed by using MEGA 4.1 software, and the reliability of the tree was assessed by bootstrap analysis with 1000 replications. The *scale bar* represents the distance unit between sequence pairs, and numbers above branches indicate neighbor-joining bootstrap values. **b** Cytopathic effect in plate-cultured MDCK cells after H_9N_2 virus infection. **c** Examination of virus particles purified from the infected MDCK cell culture medium by electron microscopy (×100,000)

Table 3 Comparison of critical amino acid residues in the hemagglutinin (HA) protein

Strain	Receptor-binding site								Cleavage motif	Potential glycosylation site						
	108	161	163	191	198	202	203	234	335–340	29–31	141–143	218–220	298–300	305–307	492–494	551–553
A/chicken/guangdong/GD01/2008	C	W	T	N	V	L	Y	Q	RSSRGL	NST	NVS	NRT	NTT	NVS	NGA	NGS
A/chicken/guangdong/GD03/2008	C	W	T	N	V	L	Y	Q	RSSRGL	NST	NVS	NRT	NTT	NVS	NGT	NGS
A/chicken/Guangdong/V/2008	C	W	T	N	V	L	Y	Q	RSSRGL	NST	NVS	NRT	NTT	NVS	NGT	NGS
A/chicken/Guangdong/TS/2004	C	W	T	N	V	L	Y	Q	RSSRGL	NST	NVS	NRT	NTT	NVS	NGT	NGS
A/Duck/Hong Kong/Y280/97	C	W	T	N	T	L	Y	L	RSSRGL	NST	NVS	NRT	NTT	NVS	NGT	–
A/Chicken/Beijing/1/94	C	W	T	N	V	L	Y	Q	RSSRGL	NST	NVT	NRT	NTT	NVS	NGT	NGS
A/Swine/Hong Kong/10/98	C	W	T	N	V	L	Y	L	RSSRGL	NST	NVS	NRT	NTT	NVS	NGT	–
A/Quail/Hong Kong/G1/97	C	W	T	H	E	L	Y	L	RSSRGL	NST	NVT	NRT	NST	NIS	NGT	–
A/Hong Kong/1073/99	C	W	T	H	E	L	Y	L	RSSRGL	NST	NVT	NRT	NST	NIS	NGT	NGS
A/Duck/Hong Kong/Y439/97	C	W	T	H	E	L	Y	Q	ASNRGL	NST	NVT	NRT	NTT	NVS	NGT	–

72 and at 96 h (Fig. 2b), suggesting that 72 h of cultivation would be sufficient for infection of MDCK cells with H_9N_2 virus.

Virus production in a bioreactor using serum-free medium

To determine the optimal time for harvesting H_9N_2 virus, MDCK cells were infected with H_9N_2 virus at 24, 48, or 72 h after seeding the cells in bioreactor, and the HA titer assays were performed at 24, 48, 72, 96, or 120 h after infection. MDCK cells cultured for only 24 h after seeding yielded the lowest virus titers (Fig. 3a). The highest virus titer was obtained from MDCK cells cultured for 72 h after seeding, with a subsequent 72-h coincubation with H_9N_2 (Fig. 3a). Typical H_9N_2 virus particles, which were purified from the highest-yielding sample, were observed by electron microscopy (Fig. S2b). Sequencing of the HA and NA genes revealed no changes in passaged viruses compared to the original isolates.

The production process which provided the highest yield of the H_9N_2 virus was further characterized. When the MDCK cell density reached its peak on the third day after seeding, the complete medium was exchanged for serum-free medium and H_9N_2 virus was added (Fig. 3b, 0 h). Following the addition of virus, a significant cytopathic effect was observed (Fig. 3b, 24–72 h). When MDCK cells were cultivated with serum-containing medium for 3 days, glucose consumption increased significantly (Fig. 3c). Glucose consumption in the bioreactor was higher than that observed for cells growing on plates (Fig. 3c), indicating the density of MDCK cells in the bioreactor was higher than that of cells growing on plates with unit volume. After adding H_9N_2 influenza virus in serum-free medium on the third day, the decrease of glucose consumption in bioreactor was more pronounced than that of cells growing on plates (Fig. 3c), implying that virus production in the bioreactor was greater than production on plates.

To investigate the impact of storage temperature on the HA titer of H_9N_2 virus, the live MDCK-derived H_9N_2 virus was stored at 4, −20, or −80 °C, respectively. Results showed that the live MDCK-derived H_9N_2 virus could be stored at 4 °C for 15 days, at −20 °C for 12 months and at −80 °C for more than 1.5 years (Fig. 3d).

Effective vaccination of chickens with MDCK-derived H_9N_2 vaccine

To determine the optimal immunizing dose of the MDCK-derived H_9N_2 vaccine in chicken, 21-day-old chickens were vaccinated and the HI assay was carried out after 28 days. The chickens vaccinated with 50 μL of MDCK-derived H_9N_2 vaccine had low HI antibody titers (lower than 6.5 $\log_2$) (Fig. 4a). Immunized chickens were challenged with GD03 for 5 days, and results showed that the H_9N_2 virus was not observed in

Table 4 Comparison of critical amino acid residues in the neuraminidase (NA) protein

Strain	Stalk deletions			Hemadsorbing site		Drug-binding pocket					
	38–39	46–50	62–64	366–373	399–404	431–433	118–119	151–152	277	292	371
A/chicken/guangdong/GD01/2008	No	No	No	IEKDSRSG	DSDNSS	PKE	RE	DR	E	R	R
A/chicken/guangdong/GD03/2008	No	No	No	IEKDSRSG	DSDNSS	PKE	RE	DR	E	R	R
A/chicken/Guangdong/V/2008	No	No	No	IEKDSRSG	DSDNSS	PKE	RE	DR	E	R	R
A/chicken/Guangdong/TS/2004	No	No	Yes	IKEDLRSG	DSDNWS	PQE	RE	DR	E	R	R
A/Duck/Hong Kong/Y280/97	No	No	Yes	IKEDSRSG	DSDNWS	PQE	RE	DR	E	R	R
A/Chicken/Beijing/1/94	No	No	No	IKKDSRSG	DSDNWS	PQE	RE	DR	E	R	R
A/Swine/Hong Kong/10/98	No	No	Yes	IKEDSRSG	DSDNWS	PQE	RE	DR	E	R	R
A/Quail/Hong Kong/G1/97	Yes	No	No	IKKDSRSG	DSDIRS	PQE	RE	DR	E	R	R
A/Hong Kong/1073/99	Yes	No	No	IKKDSRSG	DSDNWS	PQE	RE	DR	E	R	R
A/Duck/Hong Kong/Y439/97	No	No	No	ISKDSRSG	DNNNWS	PQE	RE	DR	E	R	R

chickens immunized with 200 μL of MDCK-derived H_9N_2 vaccine (Fig. 4b). Vaccine in an optimal immunization dose should be able to induce high level of antibody to neutralize the invading influenza virus, and the infection rate should be zero. These data suggest that the optimum immunizing dose was 200 μL for 21-day-old chickens.

The best immunizing dose for chickens of different ages was examined further. Chickens of various ages were vaccinated with the MDCK-derived H_9N_2 vaccine for 28 days. Seven-day-old chickens which were immunized with a dose greater than or equal to 200 μL of MDCK-derived H_9N_2 vaccine exhibited high HI antibody titers (approximately 8 $\log_2$) (Fig. 4c). The HI antibody titer of 56-day-old chickens immunized with 200 μL of the vaccine was below 6.5 $\log_2$. To elicit an appropriate HI antibody response in 56-day-old chickens, an appropriate immunizing dosage of MDCK-derived H_9N_2 vaccine was 500 μL (Fig. 4c).

Immunization with MDCK-derived H_9N_2 vaccine to defend against H_9N_2 infection in SPF chickens

Twenty-one-day-old chickens were vaccinated with the MDCK-derived H_9N_2 vaccine for 28 days, and these chickens were subsequently challenged with GD03 for 5 days. Histopathological analysis showed that both the bronchi and alveoli of untreated chickens were healthy (Fig. 5a, untreated control). In the challenge controls (nonvaccinated chickens), alveolar walls were no longer visible because there was early abscess formation (Fig. 5a, challenge control). In the chickens immunized with the MDCK-derived H_9N_2 vaccine, the structures of both bronchi and alveoli were as clear as in the untreated controls (Fig. 5a, MDCK-derived vaccine). These results indicated that lesion formation in chicken lung decreased due to vaccination treatment.

Following the H_9N_2 virus GD03 challenge, the expression of influenza virus H_9N_2 antigen in chicken lungs was

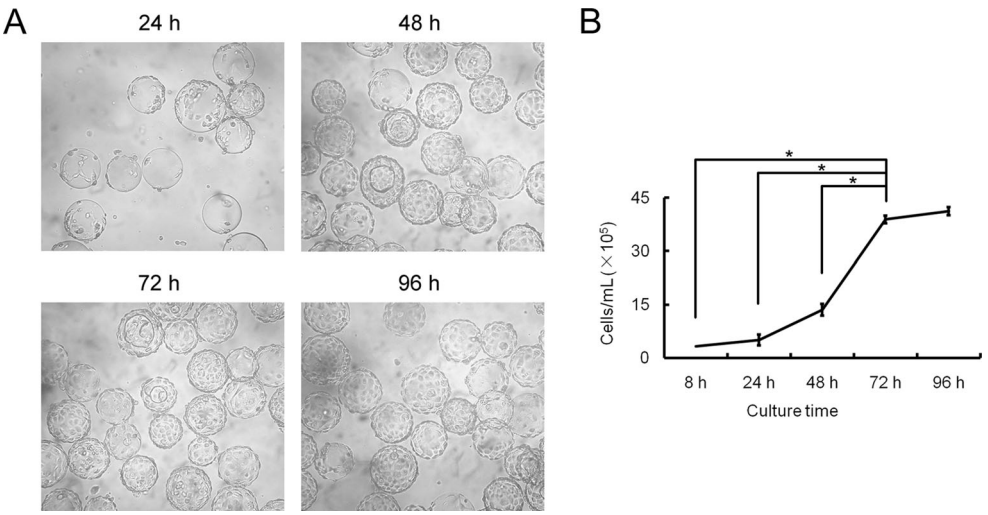

Fig. 2 The growth of MDCK cells on microcarrier beads in a bioreactor. **a** The MDCK cells cultured on microcarrier beads for 24, 48, 72, and 96 h, respectively. During the 96-h culture, sample was taken every 24 h for photomicrography. **b** Growth kinetics of MDCK cells grown in a bioreactor for 4 days. Experiments were repeated three times with three replicates, and data are shown as mean ± SD. *$p<0.05$

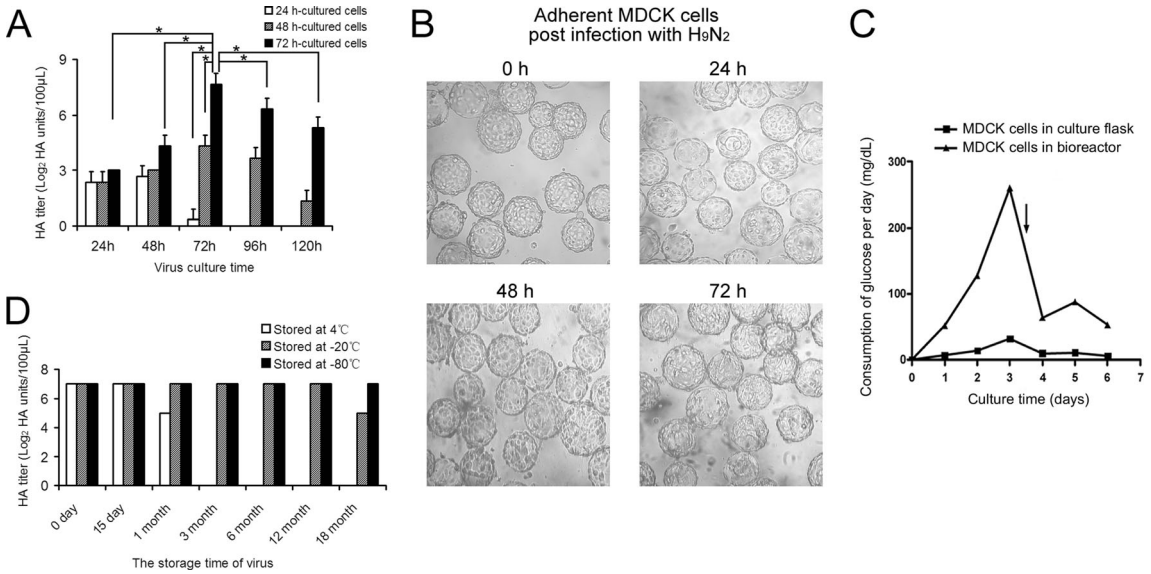

Fig. 3 Production of H_9N_2 virus from MDCK cells in a bioreactor with serum-free medium. **a** Optimal time to harvest MDCK-cultured H_9N_2 virus in the bioreactor. Data are shown as mean±SD. *$p<0.05$. **b** Cytopathic effect in MDCK cells adhered to microcarriers after H_9N_2 virus infection. **c** Glucose consumption of MDCK cells in H_9N_2 virus production. The *arrow* indicates that MDCK cells were cultured with serum-free medium following infection with H_9N_2 virus. **d** The impacts of temperature and storage time on the activity of MDCK-cultured H_9N_2 virus

evaluated by direct immunofluorescence assays. Strong influenza virus signal was detected in the challenge control (Fig. 5b). Compared with the untreated control, signals of influenza virus nucleoprotein could not be detected in lungs of chickens that were treated with MDCK-derived H_9N_2 vaccine or egg-derived H_9N_2 vaccine (Fig. 5b). Gene expression analysis using qRT-PCR demonstrated that viral gene expression in the lung, spleen, and bursa of Fabricius decreased dramatically (Fig. 5c), indicating that treatment with MDCK-derived H_9N_2 vaccine was as effective as the traditional egg-derived H_9N_2 vaccine to defend against influenza virus infection.

Enhanced immune response after immunization with MDCK-derived H_9N_2 vaccine

Twenty-one-day-old chickens were vaccinated with 200 μL of MDCK-derived H_9N_2 vaccine or 200 μL of egg-derived H_9N_2 vaccine. Chicken blood was then collected for use in the HI assay and the $CD4^+/CD8^+$ ratio test. The chickens immunized with MDCK-derived or egg-derived H_9N_2 vaccine showed a significant increase in HI antibody titer (greater than 8 $\log_2$) at 28 days post-vaccination, and no significant difference was found between the groups treated with either source of vaccine (Fig. 6a). Both vaccines also increased the $CD4^+/CD8^+$ ratio at 21 days post-vaccination. However, the increase in the

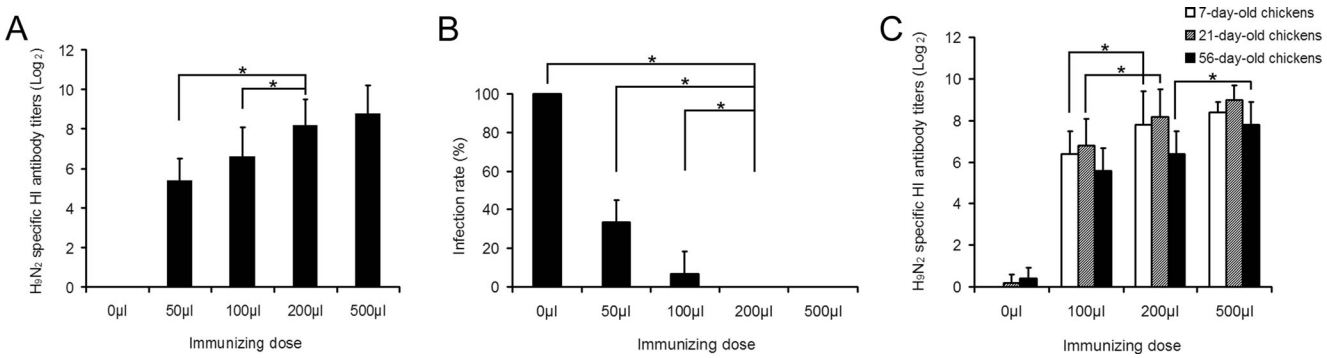

Fig. 4 Determination of the optimal immunizing dose of MDCK-derived H_9N_2 vaccine for chickens of different ages. **a** HI antibodies were induced by different doses of MDCK-derived H_9N_2 vaccine in 21-day-old SPF chickens. **b** The percentage of infection for 21-day-old SPF chickens with different immunizing doses of MDCK-derived H_9N_2 vaccine. **c** Effect of different immunizing doses of MDCK-derived H_9N_2 vaccine on SPF chickens of different ages. Data are shown as mean ± SD. *$p<0.05$

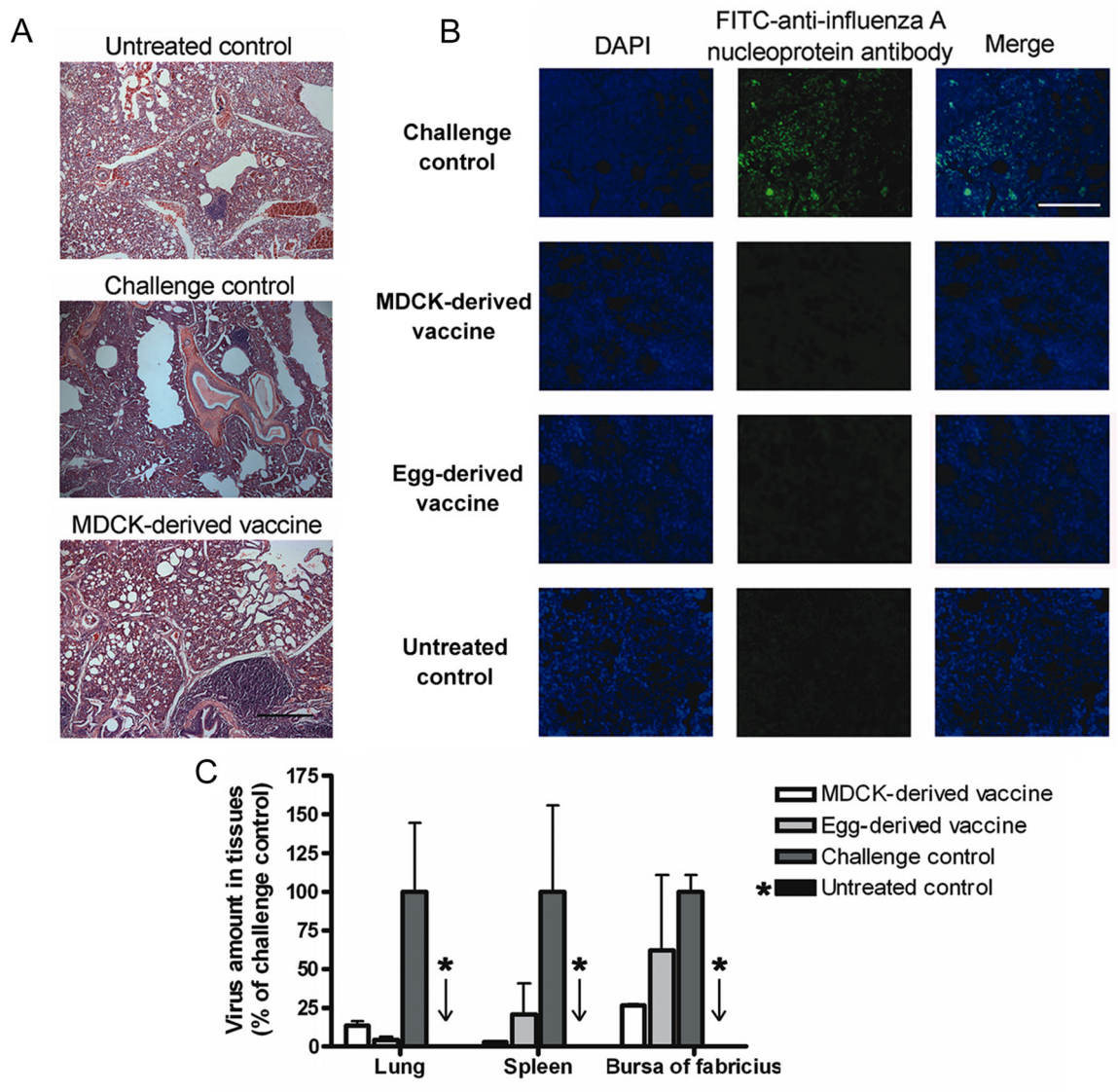

Fig. 5 Vaccination with MDCK-derived H_9N_2 vaccine protects SPF chicken from H_9N_2 virus infection. **a** Comparative pathology of chickens with or without MDCK-derived H_9N_2 vaccine immunization after H_9N_2 infection. Chickens without both vaccine immunization and H_9N_2 challenge were used as untreated controls. Unvaccinated chickens with H_9N_2 challenge were used as challenge controls. *Bar in panel* represents 200 μm. **b** MDCK-derived H_9N_2 vaccine protected chicken lung from H_9N_2 infection. *Bar in panel* represents 200 μm. **c** Vaccination with MDCK-derived H_9N_2 vaccine decreased H_9N_2 levels in chicken lung, spleen, and bursa of Fabricius. *Asterisk* indicates that the H_9N_2 virus was not detected in the untreated control

$CD4^+/CD8^+$ ratio observed in chickens vaccinated with the MDCK-derived vaccine occurred earlier than in chickens treated with the egg-derived H_9N_2 vaccine (Fig. 6b). By 28 days post-vaccination, the $CD4^+/CD8^+$ ratios of both treatment groups declined to normal levels (Fig. 6b).

On the 28th day after vaccination, chickens were challenged with GD03 for 5 days and a cytokine evaluation was performed. Compared to untreated controls, the expression of transcripts encoding five cytokines (IFN-β, IFN-γ, CXCL8, IL-18, and iNOS) was significantly upregulated in the lungs of chickens immunized with vaccine from either source (Fig. 6c). Furthermore, the transcript abundance of three cytokines (IFN-β, IFN-γ, and IL-18) was actually higher in the chickens treated with the MDCK-derived H_9N_2 vaccine than with the egg-derived H_9N_2 vaccine. ELISA was used to confirm that both IFN-γ and IL-18 were upregulated in the blood of the MDCK-derived vaccine vaccinated chickens (Fig. 6d). These data indicate that the immune response induced by the MDCK-derived H_9N_2 vaccine was as rapid and intense as the one induced by the egg-derived H_9N_2 vaccine.

Duration of immunity after MDCK-derived vaccine immunization and influence on growth

The duration of immunity for the MDCK-derived H_9N_2 vaccine was also evaluated. Twenty-one-day-old chickens

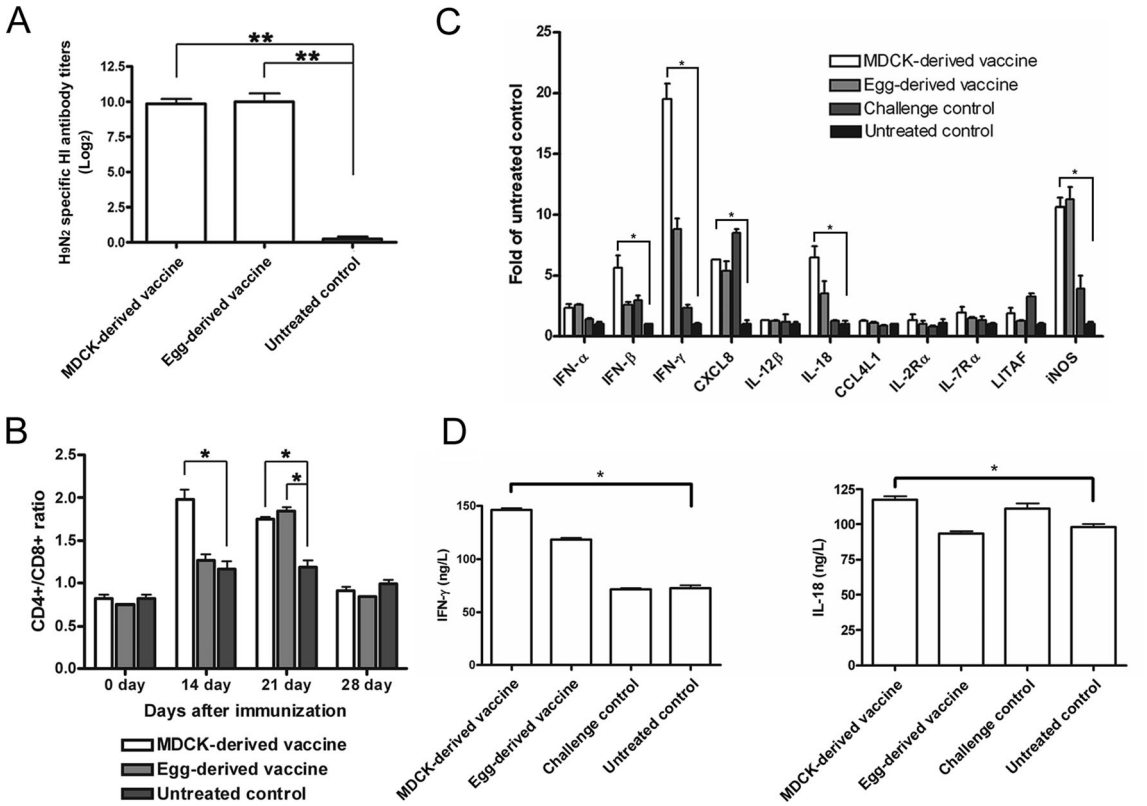

Fig. 6 MDCK-derived H_9N_2 vaccine is as effective as egg-derived H_9N_2 vaccine. **a** High level of HI antibody was induced by MDCK-derived H_9N_2 vaccine on the 28th day. **b** The activation of $CD4^+$ T cells by MDCK-derived H_9N_2 vaccine occurs earlier than in chickens treated with egg-derived H_9N_2 vaccine. **c** Expression levels of key cytokines (IFN-β, IFN-γ, and IL-18) induced by MDCK-derived H_9N_2 vaccine were higher than those induced by egg-derived H_9N_2 vaccine in chicken lung. **d** MDCK-derived H_9N_2 vaccine increased the secretion of both IFN-γ and IL-18 in serum. Data are shown as mean ± SD. $*p<0.05$; $**p<0.001$

vaccinated with MDCK-derived H_9N_2 vaccine produced HI antibody in 7 days, and the HI antibody titer was higher than 6.5 $\log_2$ by 14 days (Fig. 7a). A high level of HI antibody was maintained from 21 to 120 days post-vaccination, and the HI antibody titer fell below 6.5 $\log_2$ at 180 days post-vaccination (Fig. 7a). Following HI assays, these chickens were challenged with GD03. The average of GD03 infection rates was 26.7 % in three groups of 21-day-old chickens with vaccination for 7 days (Fig. 7b), implying that efficient immunization had not yet been established. The high levels of HI antibody produced 14 days post-vaccination protected the chickens from GD03 infection, and this protection was maintained for 5 months (Fig. 7b). One hundred eighty days following immunization, the average of GD03 infection rates in the three

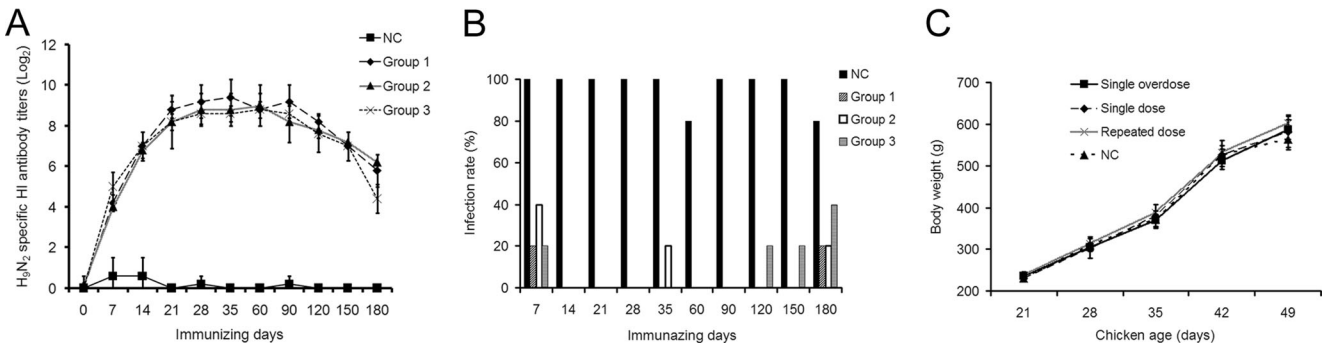

Fig. 7 MDCK-derived H_9N_2 vaccine exhibits a strong protective effect against influenza virus infection without affecting chicken growth. **a** The temporal expression pattern of HI antibodies after MDCK-derived H_9N_2 vaccine immunization. Chickens, which was nonvaccinated and uninfected but treated with PBS, was used as negative control (NC). Group 1–3 were treated in the same way that chickens were immunized with the MDCK-derived H_9N_2 vaccine. Data are shown as mean ± SD. **b** The percentage of infection for chickens after MDCK-derived H_9N_2 vaccine immunization in time-course experiments. **c** Body weight changes of chickens after MDCK-derived H_9N_2 vaccine immunization. Data are shown as mean ± SD

experimental groups was higher than 10 %, implying that the protective capacity of the MDCK-derived H_9N_2 vaccine began to diminish (Fig. 7b).

To assess whether the MDCK-derived H_9N_2 vaccine influenced chicken growth, chickens were immunized in four different ways (PBS, single dose, repeated dose, and single overdose) and changes in body weight were recorded. No significant differences in mean weight change were observed between treatments and the negative control (Fig. 7c), suggesting that administration of the MDCK-derived H_9N_2 vaccine did not influence growth.

Discussion

Over the last decade, several lineages of avian influenza H_9N_2 viruses have become endemic in Asia, causing mild respiratory disease and reductions in egg production (Cameron et al. 2000; Dong et al. 2010; Guan et al. 2000; Lee and Song 2013; Nili and Asasi 2003; Wu et al. 2008). The interspecies transmission of H_9N_2 viruses raised concerns about the possibility of increased virulence for mammals and humans (Butt et al. 2005; Cameron et al. 2000; Cong et al. 2007; Cong et al. 2008; Guan et al. 2000; Lin et al. 2000), indicating that some H_9N_2 viruses have gained the ability to replicate in mammalian cells. Early H_9N_2 isolates are mainly cultivated in egg and replicate poorly or not at all in continuous cell lines (Li et al. 2005). In this study, we present the first report on the adaptation of H_9N_2 virus to MDCK cells for producing an inactivated H_9N_2 poultry vaccine. The use of this pandemic influenza vaccine will help reducing the mild respiratory diseases in poultry, controlling the prevalence of the H_9N_2 virus, and even preventing the potential threat from the avian-to-human transmission of H_9N_2 virus.

Based on analyses of HA and NA sequences, the two novel H_9N_2 isolates with adaptation to MDCK cells belong to the LPAIV group that infects chickens. The RSSRGL motif present in the HA peptide sequence of these novel isolates suggests that they are only weakly pathogenic (Kawaoka and Webster 1988). The leucine (L) residue at position 234 within the HA peptide (H3 number 226) is a typical signature for human virus-like receptor specificity (Gambaryan et al. 2002; Ha et al. 2001; Matrosovich et al. 2001; Wan et al. 2008). At this position is a glutamine (Q) in the two H_9N_2 isolates (Table 3), implying that the two H_9N_2 isolates show a preference for binding to avian receptors. Chickens inoculated with the two H_9N_2 isolates showed clinical signs of sternutation without mortality, further confirming that the two H_9N_2 isolates are only weakly pathogenic in chickens. Based on sequence alignments and phylogenetic analyses of M1, HA, and NA genes, the virus most similar to the two H_9N_2 isolates is the A/chicken/Guangdong/V/2008 strain, which is also only mildly pathogenic in chickens (Li et al. 2012). One possible mechanism for the adaptation of the two H_9N_2 isolates to MDCK cells is the presence of K367E and W403S substitutions in the hemadsorbing site of the NA protein (Table 4). These substitutions are also present in the A/chicken/Guangdong/V/2008 strain, which is able to replicate in mice (Li et al. 2012). It has been proposed that the PB2 E627K amino acid substitution enhances H_9N_2 viral replication in mice (Wang et al. 2012), and this is also supported by the A/chicken/Guangdong/V/2008 strain (Li et al. 2012). In accordance with the new H_9N_2 isolates' similarity to the A/chicken/Guangdong/V/2008 strain, we suggest that the two H_9N_2 isolates gained a similar molecular adaptation for efficient replication in mammalian cells.

Egg-based influenza vaccine production has a successful history of supplying seasonal influenza vaccines, but egg-based vaccine production technology has many disadvantages, such as a high cost of production, potential shortages of reliable egg supplies, prolonged cultivation periods, and cumbersome operations. With increasing risk of influenza pandemic, shortages of corresponding vaccine may occur due to unreliable egg supplies. Cell-cultured influenza virus production has become a good substitute for producing vaccine in recent years. Several mammalian cell lines, such as Vero cells, PER.C6 cells, and MDCK cells can be employed to culture highly pathogenic influenza virus (Genzel et al. 2004; Genzel and Reichl 2009; Govorkova et al. 1999; Hu et al. 2008; Tree et al. 2001; Youil et al. 2004). The basic principle of the cell-based influenza vaccine manufacturing processes is that the selected cell lines are able to yield a large number of viruses and consistently high HA titers from different influenza virus strains. Based on these criteria, MDCK cells have become an attractive cell line for influenza virus cultivation (Genzel et al. 2006; Govorkova et al. 1999; Hu et al. 2011; Hu et al. 2008; Liu et al. 2009; Liu et al. 2012; Nerome et al. 1999; Tree et al. 2001). Using MDCK cells, three distinct and highly pathogenic avian influenza virus strains (H_1N_1, H_5N_1, and H_3N_2) have been adapted and cultivated (Genzel and Reichl 2009). However, there are few reports demonstrating that mildly pathogenic H_9N_2 virus is able to grow in MDCK, Vero, or PER.C6 cells. In the present study, we report that H_9N_2 virus is adapted to MDCK cells for vaccine production. The circulation of H_9N_2 viruses in the poultry industry results in great economic losses due to declines in egg production and moderate-to-high mortality (Lee and Song 2013). Our research provides a method for the rapid production of an influenza vaccine from MDCK cells to defend against influenza virus infection.

MDCK cells are typically grown on a flat surface in order to propagate influenza virus (Rott et al. 1984), but this method is technically challenging to scale for industrial production of vaccine. A promising solution to this problem is the use of microcarrier beads in bioreactor-based cell culture systems. This technique is successful because sufficient surface area

is provided by the microcarriers to achieve adherent cell growth in large-scale mammalian cell culture systems. Our results confirm that MDCK cells grow rapidly on the microcarriers. In the past few years, several studies have reported on influenza virus cultured in MDCK cells using microcarriers in a bioreactor, and the cultivation of highly pathogenic influenza virus has been explored in a series of studies (Bock et al. 2011; Genzel et al. 2004; Genzel et al. 2006; Hu et al. 2008). Due to the differences among influenza virus subtypes, it is challenging to define a consistent propagation condition for influenza viruses in MDCK cells. Here, we describe in detail a method for propagating the weakly pathogenic influenza H_9N_2 virus in MDCK cells using microcarriers in a bioreactor. To produce high-titer influenza virus of consistent quality, the storage time of the primary-cultured influenza virus was explored, thereby facilitating the manufacture of an inactivated influenza virus vaccine.

Serum is used as the source of nutrients, hormones, and growth factors for mammalian cell growth, but it is not necessary for influenza virus multiplication. Based on our observations, high-yield virus production was achieved in MDCK cells cultured with serum-free medium, which is consistent with previous reports (Bock et al. 2011; Genzel et al. 2006; Hu et al. 2011; Liu et al. 2012). In a cell-based influenza vaccine manufacturing process, the use of serum in the cell culture medium presents a few disadvantages, including the possibility of introducing contaminants (prions, mycoplamas, etc) and inducing hypersensitivity (Fishbein et al. 1993). Prior to introducing viral particles, we cultured MDCK cells with complete, serum-containing medium to facilitate cell growth. Thereafter, the serum-containing medium was exchanged for serum-free medium. The use of serum-free medium during the viral propagation step reduces the contamination risk and is convenient in reducing impurities from downstream purification steps. As most vaccine manufacturing protocols are proprietary, little information is available concerning the precise formulation of commercially available serum-free media such as Plus-MDCK medium (Hu et al. 2011). These commercial serum-free media formulations may not be suitable for the propagation of avian influenza viruses such as H_9N_2. Thus, the method described in this study can be used as a reference for producing inactivated influenza vaccine to prevent influenza pandemic.

An ideal influenza virus vaccine rapidly elicits an immune response to effectively eliminate the invading influenza virus and generate long-term immunity. Several studies concerning the cultivation of influenza virus in MDCK cells have been reported (Bock et al. 2011; Genzel et al. 2004; Genzel et al. 2006; Hu et al. 2011; Hu et al. 2008; Liu et al. 2012), but just a few experiments were performed to monitor the immunogenicity of the MDCK-derived HPAIV vaccines (Doroshenko and Halperin 2009; Palache et al. 1997). When a novel influenza virus subtype is adapted to proliferate in continuous cell lines, these parameters as well as the safety of the cell-derived inactivated influenza vaccine need to be further evaluated. Recently, two reports have presented results pertaining to the immunogenicity of inactivated MDCK-derived influenza H_5N_1 vaccine in mice (Hu et al. 2011; Liu et al. 2012). The mouse humoral immune response, which was induced by the MDCK-derived H_5N_1 vaccine, was examined using HI assay in these two studies (Hu et al. 2011; Liu et al. 2012). IFN-γ is a cytokine that is critical for innate and adaptive immunity against viral infection (Schroder et al. 2004). Our result showed that the MDCK-derived H_9N_2 vaccine not only exhibited a high titer of H_9N_2 specific HI antibodies but also induced a high level of IFN-γ expression in chickens. From this, we conclude that the MDCK-derived H_9N_2 vaccine has high immunogenicity. This result is consistent with previous experiments on MDCK-derived H_5N_1 vaccine in mice (Liu et al. 2012). Both functional antibodies and the T cell response are important for the protective capabilities of vaccines (Doherty et al. 2006). Generally, the activation of B cells required the assistance of activated $CD4^+$ T cells to differentiate into plasma cells, which secreted robust neutralization antibodies. The MDCK-derived H_9N_2 vaccine elevated the $CD4^+/CD8^+$ ratio in chicken, indicating that the $CD4^+$ T cells were effectively activated. This result is also consistent with a similar investigation of a MDCK-derived H_5N_1 vaccine in mice (Liu et al. 2012). Moreover, the duration of time required to elevate the $CD4^+/CD8^+$ ratio in chickens immunized with the MDCK-derived H_9N_2 vaccine was shorter than that with an egg-derived H_9N_2 vaccine, suggesting that the MDCK-derived H_9N_2 vaccine possessed immunogenicity as high as the egg-derived source. This conclusion was further supported by the fact that the MDCK-derived H_9N_2 vaccine induced a more robust expression of cytokines such as IFN-γ and IL-18.

The MDCK-derived H_9N_2 vaccine effectively induced an immune response to prevent against influenza virus infection, but this result is only partially sufficient to evaluate the safety of this vaccine. Histopathological analysis showed that the MDCK-derived H_9N_2 vaccine protected chicken lung from the cytotoxic effects of pathogenic influenza virus infection. Furthermore, immunization using the MDCK-derived H_9N_2 vaccine is effective in eliminating influenza viral particles in chicken lung. In other organs, such as the spleen and bursa of Fabricius, the effectiveness of the MDCK-derived H_9N_2 vaccine in reducing influenza virus replication was even more pronounced than the egg-derived H_9N_2 vaccine. These data suggest that the MDCK-derived influenza vaccine is a good substitute for the egg-derived influenza vaccine to protect chickens from influenza virus infection. The MDCK-derived H_9N_2 vaccine also did not affect chicken growth, and chickens thus immunized maintained high titers of H_9N_2-specific HI antibody for 5 months. Taken together with previous reports (Liu et al. 2012), our results demonstrate that the MDCK-

derived influenza vaccine is a safe choice for the prevention of influenza pandemics in the poultry industry.

In summary, the current study demonstrates that the low pathogenic influenza H_9N_2 virus is able to proliferate in a large-scale MDCK cell culture system to rapidly produce a safe H_9N_2 vaccine for protecting chickens against influenza virus infection. This method will be valuable for industrial vaccine manufacturing due to its low cost and ability to prevent LPAIV outbreaks in poultry. Mutations in the direct viral descendants of H_9N_2 virus adapted to MDCK cells should be monitored to detect the occurrence of antigentic drift and shift. Furthermore, the molecular mechanisms of mammalian adaptation and interspecies transmission of H_9N_2 avian influenza viruses should be further explored. When an influenza virus is isolated in relation to an influenza pandemic, microcarrier-anchored MDCK cells are an ideal choice to propagate the isolated influenza virus and to manufacture an inactivated influenza vaccine rapidly. As MDCK cells have been conversed to suspension culture (Chu et al. 2009; van Wielink et al. 2011), further work will be extended to perform investigations for the cultivation of different influenza virus strains in suspension MDCK cells.

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Conflict of interest The authors have declared that no competing interests exist.

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