CHINESE JOURNAL OF BIOTECHNOLOGY
Volume 22, Issue 5, September 2006
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
SCIENCE DIRECT®

Cite this article as: Chin J Biotech, 2006, 22(5), 720−726.

RESEARCH PAPER

Generation of High-yield Vaccine Strain Wholly Derived from Avian Influenza Viruses by Reverse Genetics

LIU Ming[1,*], ZHANG Yun[1], LIU Chun-Guo[1], PAN Wei-Qi[1,2], LIU Chao-Nan[1,2], YANG Tao[1]

1 National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of CAAS, Harbin 150001, China

2 College of Life Science Technologies, Northeast Agricultural University, Harbin 150030, China

Abstract: Highly pathogenic avian influenza A (HPAI) viruses of the H5N1 subtypes caused enormous economical loss to poultry farms in China and Southeastern Asian countries. The vaccination program is a reliable strategy in controlling the prevalence of these disastrous diseases. The six internal genes of the high-yield influenza virus A/Goose/Dalian/3/01 (H9N2), the haemagglutinin (HA) gene of A/Goose/HLJ/QFY/04 (H5N1) strain, and the neuraminidase gene from A/Duck/Germany/1215/73 (H2N3) reference strain were amplified by RT-PCR technique. The HA gene was modified by the deletion of four basic amino acids of the connecting peptide between HA1 and HA2. Eight gene expressing plasmids were constructed, and the recombinant virus rH5N3 were generated by cell transfection. The infection of chicken embryos and the challenge tests involving chickens demonstrated that the recombinant H5N3 (rH5N3) influenza virus is avirulent. The allantoic fluids of rH5N3-infected eggs contain high-titer influenza viruses with haemagglutination unit of 1:2 048, which are eight times those of the parental H5N1 virus. The rH5N3 oil-emulsified vaccine could induce haemagglutination inhibition (HI) antibodies in chickens in 2 weeks post-vaccination, and the maximum geometric mean HI-titers were observed 4–5 weeks post-vaccination and were kept under observation for 18 weeks. The rH5N3-vaccinated chickens were fully protected against morbidity and mortality of the lethal challenge of the H5N1 HPAI viruses, A/Goose/Guangdong/1/96 and A/Goose/HLJ/QFY/04, which had 8 years expansion and differences among multiple amino acids in HA protein. The N3 neuraminidase protein marker makes it possible to distinguish between H5N1-infected and H5N3-vaccinated animals.

Key Words: avian influenza virus; reverse genetics; high yield strain; vaccine evaluation

Highly pathogenic avian influenza (HPAI), a highly infectious and systemic disease affecting chickens, with a mortality as high as 100 %, is restricted to subtypes H5 and H7, although not all viruses of these subtypes necessarily cause HPAI. Avian influenza viruses (AIV) can infect chickens, turkeys, pheasants, quails, ducks, geese, and guinea fowls, as well as a wide variety of other birds[1]. The appearance of H5N1 HPAI in wild and captive birds in 2003 suggests a possible change in the ecology of the virus and a need for examination of wild aquatic birds as reservoirs and disseminators of H5N1 virus. H5N1 HPAI virus has been spreading rapidly in chicken flocks and wild birds in Southeast Asia ever since its first emergence in southern China in 1996. Between late 2003 and early 2004, outbreaks of highly pathogenic avian H5N1 influenza occurred among poultry in eight Asian countries, causing the death of tens of millions of birds[2]. The H5N1 HPAI virus is spreading to poultry or other birds in European, African and American continents[3,4]. HPAI viruses of the H5N1 subtypes not only lead to enormous economical damage to poultry industry but also pose a serious threat to public health[5]. The vaccination program is a reliable strategy in controlling the prevalence of

Received: March 27, 2006; Accepted: May 17, 2006.
* Corresponding author. Tel: +86-451-85935069; E-mail: liuming04@126.com
This work was supported by the grant from the National High Technology Research and Development Program of China (863 Program) (No. 2003AA241110).

these disastrous diseases. As of February 2006, 169 cases of human H5N1 infection have been confirmed in Indonesia, Vietnam, Thailand, Cambodia, and China, of which 91 were fatal, according to the World Health Organization. Of these, eight people died, and 12 were confirmed cases in China.

The virions are spherical. There are two kinds of glycoproteins on the surface of the virions: homotrimers of the haemagglutinin (HA) protein and homotetramers of neuraminidase protein (NA). These glycoproteins, and HA, in particular, have been recognized as key antigens in the host response to influenza virus infection and vaccination. HA is the major virus antigen that induces the protective immunological reaction. HA plays an important role in virus infection process[6]. The cleavage site of the HA protein and the motifs of multibasic amino acids in the connecting peptide between HA1 and HA2 is associated with the systemic spread of virus and high pathogenicity in chickens[7,8]. Protective immunity against avian influenza is based mainly on the presence of the virus neutralizing antibodies against the HA protein, which is associated with the haemagglutination inhibition activity. However, immunity pressure could cause influenza virus HA gene mutation, thereby resulting in the failure of the vaccine, and therefore the mutant prevails.

The major methods that were attempted to control the emergence the HPAI include the slaughtering of infected poultry flocks and improvement of biosecurity. However, the use of inactivated vaccine for the prevention and control of HPAI may be the most economical means for the poultry farms in southeastern Asian countries, with a range of different husbandry management levels.

The classical reassortment method could not be applied to the selection of H5N1 influenza vaccine strain[9]. The established influenza virus PR8-based reverse-genetics system makes the procedure for high-growth vaccine strain generation simple and easy[10]. Neumann[11] first reported the generation of influenza virus from 17-plasmids reverse-genetics systems in 1999. Hoffmann[12] established an eight-plasmids reverse-genetics system and generated H1, H3, H6, and H9 subtypes influenza virus strains. Liu[13] reported the generation of PR8-based high-yield H5N3 vaccine strain by reverse genetics. In this study, we established a novel influenza reverse-genetics system, and all the high-growth properties were derived from avian influenza virus so as to enhance the biosafety of influenza vaccine. The rescued H5N3 vaccine virus grows to high titers in eggs and MDCK cells, bears N3 protein molecular markers, has a better HA gene match with the currently prevalent viruses.

1 Materials and methods

1.1 Virus strains and cells and plasmids

The A/Goose/HLJ/QFY/2004 (H5N1; QFY) strain, which was isolated from a diseased goose, a reference A/Duck/Germany/1215/73 (H2N3) avian influenza strain, and an avirulent high-growth A/Goose/Dalian/3/2001 (H9N2; Dalian, China) strain were diluted in phosphate buffered saline (PBS), at $1:10^3$ and injected into the allantoic cavity of 10-day-old embryonated chickens' eggs, propagated at 37 °C for 28–48 h, after which the allantoic fluids containing virus were harvested and stored at −70 °C until use.

Madin-Darby Canine kidney (MDCK) cells were cultured in MEM (GIBCO BRL) supplemented with 10 % FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 1.5 mg/mL sodium bicarbonate, 10 mmol/L Hepes, and nonessential amino acids. 293T cells were cultured in DMEM (Hyclone) supplemented with 10 % FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mmol/L glutamine, 1 mmol/L sodiumpyruvate, and nonessential amino acids. The transcription and translation bidirectional vector pHW2000 was a gift from Dr. Webster (St. Jude Children's Research Hospital, USA).

1.2 Reagents and kits

Trizol Reagents for viral RNA extraction was purchased from Invitrogen (Shanghai, China). AMV and Ex Taq DNA Polymerase were products of TaKaRa Biotech Co. Ltd (Dalian, China). Restriction endonucleases *Bsm*B I, *Bsa* I and T4 DNA ligase were products of New England Biolabs Inc. Restriction endonuclease *Aar* I was obtained from Fermentas. Plasmids mini kit, Gel extraction mini kit, and PCR purification mini kit were purchased from Watson Biotechnologies Inc (Shanghai, China). Lipofactamine 2000, OPTI-MEM I, and DMEM cell culture media were purchased from Invitrogen.

1.3 Primers

The reverse-transcription universal primer Uni-12: 5′-AGCAAAAGCAGG-3′ was synthesized according to influenza sequences. Primers of oligonucleotides were designed for the amplification of genes of polymerases of A/Goose/Dalian/3/2001, the NP, NA M NS gene-specific primers were commercially synthesized (Invitrogen) as reported (Hoffmann *et al*). The oligonucleotides are listed in Table 1. Those primers were also used to characterize the rescued viruses by RT-PCR.

1.4 RNA extraction, cDNA synthesis and RT-PCR

The Trizol Reagent (Invitrogen) was used in this study to extract viral RNA from 400 μL of allantoic fluids from infected embryonated eggs according to manufacturer's instructions. In brief, the RNA was transcribed into cDNA using AMV reverse transcriptase (TaKaRa) according to the protocol provided, using 0.5 μg of Uni12. The RT reaction was performed at 42 °C for 60 min. One microliter of the RT-reaction was used for each PCR reaction.

To increase the amplifying efficiency of P-gene segments, two partial PCRs that spanned the full length of one gene segment was applied. The cDNA was amplified using the Ex Taq (TaKaRa) according to the protocols provided. The first cycle of the amplification program consisted of a 4-min period