

Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics

Guobin Tian^a, Suhua Zhang^b, Yanbing Li^a, Zhigao Bu^c, Peihong Liu^b, Jinping Zhou^b,
Chengjun Li^{a,c}, Jianzhong Shi^a, Kangzhen Yu^{a,1}, Hualan Chen^{a,c,*}

^aAnimal Influenza Laboratory of the Ministry of Agriculture, Harbin Veterinary Research Institute,
Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, PR China

^bShanghai Animal Husbandry and Veterinary Station, 30 855 Lane, Hongjing Road, Shanghai 201103, PR China

^cNational Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute,
Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, PR China

Received 14 June 2005; returned to author for revision 5 July 2005; accepted 14 July 2005

Available online 9 August 2005

Abstract

We generated a high-growth H5N1/PR8 virus by plasmid-based reverse genetics. The virulence associated multiple basic amino acids of the HA gene were removed, and the resulting virus is attenuated for chickens and chicken eggs. A formalin-inactivated oil-emulsion vaccine was prepared from this virus. When SPF chickens were inoculated with 0.3 ml of the vaccine, the hemagglutinin-inhibition (HI) antibody became detectable at 1 week post-vaccination (p.v.) and reached a peak of 10log₂ at 6 weeks p.v. then slowly declined to 4log₂ at 43 weeks p.v. Challenge studies performed at 2, 3 and 43 weeks p.v. indicated that all of the chickens were completely protected from disease signs and death. Ducks and geese were completely protected from highly pathogenic H5N1 virus challenge 3 weeks p.v. The duration of protective immunity in ducks and geese was investigated by detecting the HI antibody of the field vaccinated birds, and the results indicated that 3 doses of the vaccine inoculation in geese could induce a 34 weeks protection, while 2 doses induced more than 52 weeks protection in ducks. We first reported that an oil-emulsion inactivated vaccine derived from a high-growth H5N1 vaccine induced approximately 10 months of protective immunity in chickens and demonstrated that the oil-emulsion inactivated avian influenza vaccine is immunogenic for geese and ducks. These results provide useful information for the application of vaccines to the control of H5N1 avian influenza in poultry, including chickens and domestic waterfowl.

© 2005 Elsevier Inc. All rights reserved.

Keywords: H5N1 influenza; Reverse genetics vaccine; Chickens; Ducks; Geese

Introduction

An H5N1 avian influenza virus A/goose/Guangdong/1/96 (GSGD/96) was first isolated from geese in Guangdong province in China in 1996 (Chen et al., 2004; Xu et al.,

1999). In 1997, H5N1 avian influenza virus caused disease outbreaks in poultry in Hong Kong (Sims et al., 2003; Shortridge et al., 1998), and a reassortant virus bearing the hemagglutinin (HA) gene of the GSGD/96-like virus and the NA gene and 6 internal genes from H6N1-subtype A/teal/Hong Kong/W312/97-like virus (Chin et al., 2002) was transmitted into humans and caused 6 deaths in 18 infected people (Claas et al., 1998; Subbarao et al., 1998). In early February of 2003, H5N1 virus reemerged in a family in Hong Kong (Anonymous, 2003; Peiris et al., 2004). Starting from late 2003, H5N1 influenza viruses began to spread and caused disease outbreaks in China, Japan (Mase et al., 2005), South Korea (Lee et al., 2005), Thailand, Vietnam, Indo-

* Corresponding author. Animal Influenza Laboratory of the Ministry of Agriculture, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, PR China. Fax: +86 451 82733132.

E-mail address: hlchen1@yahoo.com (H. Chen).

¹ Current address: National Animal Husbandry and Veterinary Service of the Ministry of Agriculture, 20 Maizidian Street, Beijing 100026, PR of China.

nesia, Cambodia, Malaysia and Laos, resulting in the destruction of hundreds of millions of poultry, including chickens, ducks and geese. In Thailand (Puthavathana et al., 2005), Vietnam (Tran et al., 2004) and Cambodia, the viruses were transmitted into humans and currently have caused death in 50 of the 80 confirmed cases. H5N1 virus also caused disease and death in tigers and leopards in Thailand (Keawcharoen et al., 2004; Thanawongnuweeh, 2005). These facts emphasize that the H5N1 viruses are not only pathogens disastrous for domestic poultry but also bear a substantial threat to public health.

Wild birds are regarded as natural reservoirs for avian influenza viruses. Free-range domestic waterfowl have chances to contact with both wild birds and domestic animals, including poultry and mammals, such as pigs, and therefore can function as intermediate hosts to transmit the avian influenza viruses from wild birds to other hosts. H5N1 avian influenza viruses have circulated in domestic waterfowl in certain southeast Asia countries for a number of years (Chen et al., 2004; Nguyen et al., 2005; Webster et al., 2002), and these waterfowl usually do not show any disease signs, even when they carry viruses that are highly pathogenic for chickens (Alexander et al., 1986; Chen et al., 2004; Perkins and Swayne, 2002; Webster et al., 2002). If active surveillance is not rigorously carried out, these viruses will not be detected unless they cause disease outbreaks or are transmitted to sensitive hosts, such as chickens or turkeys. It is quite common in southern China and other Asia countries that pigs and ducks are housed in close proximity, especially in farming villages, where families typically own a small number of pigs and ducks. This proximity creates the opportunity for viruses to transmit between ducks and pigs and to adapt to mammalian hosts. Therefore, to prevent waterfowl from H5N1 avian influenza infection would likely cut the transmission chain of these viruses and greatly enhance efforts to control and prevent disease outbreak in other poultry and animals, including humans.

The culling of infected poultry is the time-honored method to control or eradicate the highly pathogenic avian influenza outbreaks and also the best-known way to prevent transmission to humans. However, when the viruses are widely spread over a huge area and involved in multiple avian species, culling and physical containment are highly unlikely to be successful. An alternative strategy would be culling plus vaccination.

Egg-grown inactivated influenza vaccines have been used for humans for many years and also for controlling avian influenza in chickens and turkeys in some countries (Abraham et al., 1988; Ellis et al., 2004; Karunakaran et al., 1987; Capua et al., 2004), however, there is a lack of studies on waterfowl vaccination. The ideal seed virus for vaccine production is a strain of low pathogenicity that is well-matched antigenically with the prevailing virus and capable of growing well in eggs, which is crucial to mass production (Kilbourne, 1969; Chen et al., 2003). All H5N1 viruses isolated in China since 1996 have multiple basic amino

acids in the cleavage site of HA and therefore are all either highly pathogenic or potentially highly pathogenic avian influenza viruses unsuitable for vaccine production. The main problems are the requirement for high-level bio-containment facilities to adequately handle these viruses and the inability to obtain high yields of virus in embryonated chickens' eggs (Richmond and McKinney, 1993; Subbarao and Katz, 2004; Takada et al., 1999; Zambon, 1998).

Plasmid-based reverse genetics, developed in the late 1990s (Fodor et al., 1999; Garcia-Sastre and Palese, 1993; Hoffmann et al., 2000a,b; Neumann et al., 1999), is a powerful tool to generate ideal reassortant influenza vaccine candidates (Hoffmann et al., 2002; Lipatov et al., 2005; Marsh and Tannock, 2005; Nicolson et al., 2005; Schickli et al., 2001; Webby et al., 2004). Previous studies indicated that the A/goose/Guangdong/1/96 (GSGD/96) virus is the HA gene donor of the avian influenza viruses circulating in the poultry in China (Chen et al., 2004), and the HA gene of GSGD/96 virus has been proved to be very immunogenic in the recombinant fowlpox vaccine and DNA vaccine studies (Qiao et al., 2003; Chen et al., 2001). In this study, we generated a low pathogenicity H5N1 reassortant virus that derives its HA and NA genes from GSGD/96 virus and 6 internal genes from the high-growth A/Puerto Rico/8/34 (PR8) virus by plasmid-based reverse genetics, as described previously (Subbarao et al., 2003). The biological properties of the virus were characterized, and the efficacy of a formalin-inactivated vaccine derived from the virus was evaluated. Antibody detection and challenge studies indicated that one dose of the vaccine preparations was able to induce 10 months of protective immune response in SPF chickens under the experimental conditions. We also demonstrated that this inactivated vaccine is immunogenic in ducks and geese and is able to completely protect these waterfowl from highly pathogenic H5N1 virus challenge.

Results

Generation of a reassortant H5N1/PR8 virus

GSGD/96 is the first H5N1 AIV isolated in China in 1996, and phylogenetic data suggested that the GSGD/96-like virus is the HA gene donor of the Hong Kong 97 H5N1 human influenza viruses. Avian influenza surveillance has been conducted in China since 1996, and a series of H5N1 AIV strains have been isolated from health ducks during the last several years. However, the antigenicity of these isolates was similar to the GSGD/96 (the heterologous HI titers were within a 2-fold range when compared with the homologous titers of these viruses) (Chen et al., 2004). Therefore, we selected the GSGD/96 as the HA and NA donor to generate the reassortant virus. The multiple basic amino acids of the HA cleavage site (RERRRKKR↓GLF) that are associated with the virulence of the H5 avian influenza virus in chickens and mammals were changed into RETR↓GLF, a

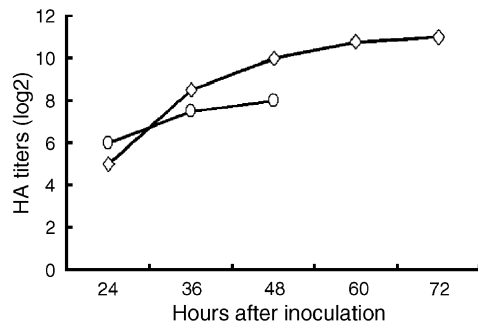


Fig. 1. Growth properties of H5N1 viruses in embryonated eggs. 0.1 ml of 100EID₅₀ of the Re-1 reassortant virus (◇) or wild-type GSGD/96 virus (○) were inoculated into the allantoic cavities of 10-day-old embryonated eggs, and the allantoic fluid of five eggs in each group was harvested at the time points of 24, 36, 48, 60 and 72 h post-inoculation and pooled for checking the HA titers.

characteristic of low pathogenicity avian influenza viruses (Hatta et al., 2001; Perdue et al., 1997; Senne et al., 1996). A PR8-based reassortant virus, A/Harbin/Re-1/2003 (Re-1), which contains the HA and NA genes of GSGD/96, was generated by plasmid-based reverse genetics, and the genotype was confirmed by sequencing the full length of the HA and NA genes and part of each internal gene fragment. Antigenic analysis revealed that Re-1 bears the same antigenicity as GSGD/96. Plaque assay performed in MDCK cells indicated that Re-1 lost the ability to form plaque in the absence of TPCK trypsin, which is consistent with the results of previous reports (Liu et al., 2003a; Subbarao et al., 2003).

In vitro growth properties

Chicken eggs produced whole virus inactivated vaccines were used for prevention of influenza in humans, poultry and other animals. The high growth property of the vaccine strain is very important for mass production of the vaccine. Ten-day-old embryonated chicken eggs were inoculated with 100 EID₅₀ of Re-1 or GSGD/96 virus, the viruses were harvested at different time points, and the HA titers were checked (Fig. 1). The wild-type GSGD/96 killed all of the eggs within 24–48 h, and the titers stopped increasing after that. However, the reassortant virus Re-1 did not kill eggs even 72 h after inoculation, and the HA titers reached 11log₂ (Fig. 1).

Pathotyping and replication in chickens

Chickens inoculated with the Re-1 virus did not exhibit disease signs or death during the observation period. A very low titer of virus shedding (from undiluted samples) was detected from 3 of 10 chickens in the oropharyngeal swabs on day 3 post-inoculation, and 7 of 10 chickens had seroconverted by day 14 p.i. All chickens in the GSGD/96 inoculated group died during the observation period, and the average titers of the shedding viruses were 2.5–4.5

log₁₀EID₅₀ from the oropharynx and 2.8–3.5 log₁₀EID₅₀ from the cloacae.

Immunogenicity and protective efficacy of Re-1 formalin-inactivated vaccine in chickens

0.3 ml of formalin-inactivated vaccines (containing 2.8 μg of the HA protein) prepared from the Re-1 virus were i.m. injected into 3-week-old SPF chickens, and sera was collected on a weekly base to check the dynamic changes in the HI antibody titer. As shown in Fig. 2, the HI antibody was detected at 1-week post-vaccination (p.v.) and reached the peak of 10log₂ at 6 weeks p.v. then very slowly declined to 4log₂ at 43 weeks p.v. (Fig. 2).

Our unpublished data indicated that, when the HI antibody titers of chickens increased to 4log₂ or higher at 2 or 3 weeks after vaccination, the chickens were completely protected from virus challenge. To determine whether the long-lasting HI antibody of the immunized chickens still correlated with protection, groups of chickens were challenged with the homologous highly pathogenic virus GSGD/96 at the different time points of 2, 3 and 43 weeks p.v., respectively. The results shown in Table 1 indicate that the vaccinated chickens were completely protected from the highly pathogenic homologous virus GSGD/96 challenge at 2, 3 and 43 weeks p.v., although very low titers of virus (from undiluted samples) were recovered from the oropharyngeal swabs of one chicken at day 3 when challenged at 2 weeks p.v. (Table 1). Chickens in the control groups shed virus from both oropharynx and cloaca and died prior to day 6 after challenge (Table 1). These results demonstrate that, after one dose inoculation, chickens were protected from highly pathogenic virus challenge for at least 43 weeks (10 months).

To evaluate the protective efficacy of the Re-1 vaccine against the H5N1 avian influenza viruses isolated in 2004, we challenged the chickens at 3 weeks p.v. with A/chicken/Tianjing/65/2004 (H5N1) (CKTJ/04) and A/duck/Shanghai/16/2004 (H5N1) (DKSH/04), respectively. The results in Table 1 show that the chickens were completely protected from death and disease, although lower titers of virus

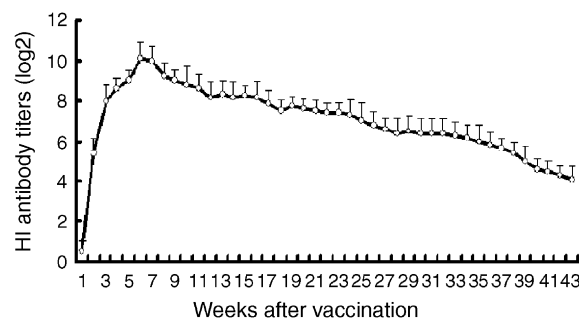


Fig. 2. HI antibody duration induced by inactivated vaccine derived from Re-1 strain in SPF chickens. Three-week-old white Leghorn SPF chickens were injected intramuscularly (i.m.) with 0.3 ml of formalin-inactivated vaccine, and sera were collected randomly from 8 chickens on a weekly base for HI antibody detection. The bars indicate the standard deviation.

Table 1
Protective efficacy of the H5N1 formalin-inactivated vaccines in SPF chickens

| Administration ^a | | | Virus isolation from the swabs on different days p.c.: shedding/total (log ₁₀ EID ₅₀) | | | | | | Survival/total |
|-----------------------------|-----------------|-----------------------------|--|-----------------|-----------------|-----------------|---------------|--------------|----------------|
| Vaccines | Challenge virus | Challenge time (weeks p.v.) | Day 3 | | Day 5 | | Day 7 | | |
| | | | Oropharyngeal | Cloacal | Oropharyngeal | Cloacal | Oropharyngeal | Cloacal | |
| <i>Experiment I</i> | | | | | | | | | |
| Re-1 | GSGD/96 | 2 | 1/8 (0.9) | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 |
| Control | GSGD/96 | 2 | 8/8 (3.1 ± 0.6) | 8/8 (2.4 ± 1.2) | 5/5 (2.4 ± 0.6) | 5/5 (2.9 ± 0.5) | ^b | ^b | 0/8 |
| Re-1 | GSGD/96 | 3 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 |
| Control | GSGD/96 | 3 | 8/8 (3.3 ± 0.9) | 8/8 (2.2 ± 1.3) | 6/6 (2.4 ± 0.7) | 6/6 (2.7 ± 0.6) | ^b | ^b | 0/8 |
| Re-1 | GSGD/96 | 43 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 |
| Control | GSGD/96 | 43 | 8/8 (3.1 ± 0.6) | 8/8 (2.6 ± 0.6) | 1/1 (1.9) | 1/1 (1.4) | ^b | ^b | 0/8 |
| <i>Experiment II</i> | | | | | | | | | |
| Re-1 | CKTJ/04 | 3 | 2/8 (1.0 ± 0.9) | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 |
| Control | CKTJ/04 | 3 | 8/8 (4.1 ± 0.5) | 8/8 (3.8 ± 0.8) | ^b | ^b | ^b | ^b | 0/8 |
| Re-1 | DKNH/04 | 3 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 8/8 |
| Control | DKNH/04 | 3 | 8/8 (3.9 ± 0.7) | 8/8 (4.3 ± 0.3) | ^b | ^b | ^b | ^b | 0/8 |

^a Groups of 3-week-old SPF chickens were vaccinated with 0.3 ml of the vaccine preparations and challenged with GSGD/96 at different time points of 2, 3, 26 and 43 weeks post-vaccination, respectively, in experiment I. In experiment II, groups of vaccinated chickens were challenged with two H5N1 avian influenza viruses isolated in China in 2004 at 3 weeks p.v.

^b Chickens died.

shedding were detected from the oropharyngeal swabs of 2 of the 8 chickens challenged with CKTJ/04 virus. All chickens in the control groups shed high titers of virus through both oropharynx and cloaca and died within 2 days post-challenge (Table 1).

The vaccine efficacy in geese

Laboratory studies

Three-week-old geese were immunized with 0.5 ml of the vaccine preparation, and only 2 of 5 geese survived from challenge with highly pathogenic virus at 2 weeks after

immunization, with virus shedding from both oropharynx and cloaca detected on days 3, 5 and 7 after challenge. However, the geese were completely protected from challenge at 3 weeks after vaccination, none of the geese shed virus, no disease signs were observed, and no geese died. All geese in the control group died within 7 days after challenge, and virus shedding was detected on days 3, 5 and 7 (Table 2).

Field studies

The HI antibody duration in the geese vaccinated in the field is shown in Fig. 3a. During the 36 weeks investigation

Table 2
Vaccine efficacy of the H5N1-inactivated vaccine in geese and ducks

| Administration ^a | | | Virus isolation from the swabs collected on different days after challenge: | | | | | | Survival/total |
|-----------------------------|--------------------------------|-----------------------------|---|------------------|------------------|------------------|-----------------|-----------------|----------------|
| Animals | Group and vaccination schedule | Challenge time (weeks p.v.) | Day 3 | | Day 5 | | Day 7 | | |
| | | | Oropharyngeal | Cloacal | Oropharyngeal | Cloacal | Oropharyngeal | Cloacal | |
| Geese ^a | Vaccinated | 2 | 1/5 (0.9) | 0/5 (<) | 4/5 (2.0 ± 1.4) | 3/5 (0.8 ± 0.4) | 1/3 (2.0) | 2/3 (2.1 ± 1.4) | 3/5 |
| | Control | 2 | 3/5 (1.6 ± 0.3) | 0/5 (<) | 5/5 (2.4 ± 0.1) | 3/5 (1.8 ± 1.0) | ^b | ^b | 0/5 |
| | Vaccinated | 3 | 0/5 (<) ^c | 0/5 (<) | 0/5 (<) | 0/5 (<) | 0/5 (<) | 0/5 (<) | 5/5 |
| | Control | 3 | 0/5 (<) | 1/5 (1.5) | 5/5 (1.7 ± 0.4) | 3/5 (1.1 ± 0.5) | 2/2 (2.4 ± 0.2) | 2/2 (1.7 ± 0.3) | 0/5 |
| Field geese | Vaccinated | 34 ^d | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 | 10/10 |
| | Control | ^e | 9/10 (1.4 ± 1.1) | 4/10 (0.9 ± 0.7) | 9/9 (3.2 ± 0.7) | 3/9 (0.9 ± 0.7) | 1/1 (3.3) | ^b | 0/10 |
| Ducks ^a | Vaccinated | 3 | 0/30 (<) | 0/30 (<) | 0/30 (<) | 0/30 (<) | 0/30 (<) | 0/30 (<) | 30/30 |
| | Control | 3 | 13/15 (2.1 ± 0.8) | 8/15 (1.8 ± 0.6) | 7/15 (2.1 ± 1.0) | 5/15 (0.8 ± 0.3) | 0/2 (<) | 0/2 (<) | 2/15 |
| Field ducks | Vaccinated | 51 ^f | 2/10 (0.6 ± 0.1) | 0/10 (<) | 0/10 (<) | 0/10 (<) | 0/10 (<) | 0/10 (<) | 10/10 |
| | Control | ^g | 10/10 (3.0 ± 0.6) | 3/10 (1.2 ± 1.1) | 5/9 (1.8 ± 1.2) | 1/9 (0.7 ± 0.5) | 0/9 (<) | 0/9 (<) | 9/10 |

^a 3-week-old avian influenza serological negative geese or ducks were vaccinated with 0.5 ml of the Re-1 vaccine preparations and were challenged with 10^{7.5}EID₅₀ of the highly pathogenic virus DKSH/04 in 0.1 ml volume intranasally at 2 or 3 weeks p.v.

^b All geese in that group died.

^c “<” means virus was not detected from undiluted samples.

^d Geese were challenged at 34 weeks after the first shot (20 weeks after the third shot).

^e Eight-month-old avian influenza negative geese were used as control.

^f Ducks were challenged 52 weeks after the first shot (38 weeks after the second shot).

^g Ten-month-old avian influenza negative ducks were used as control.

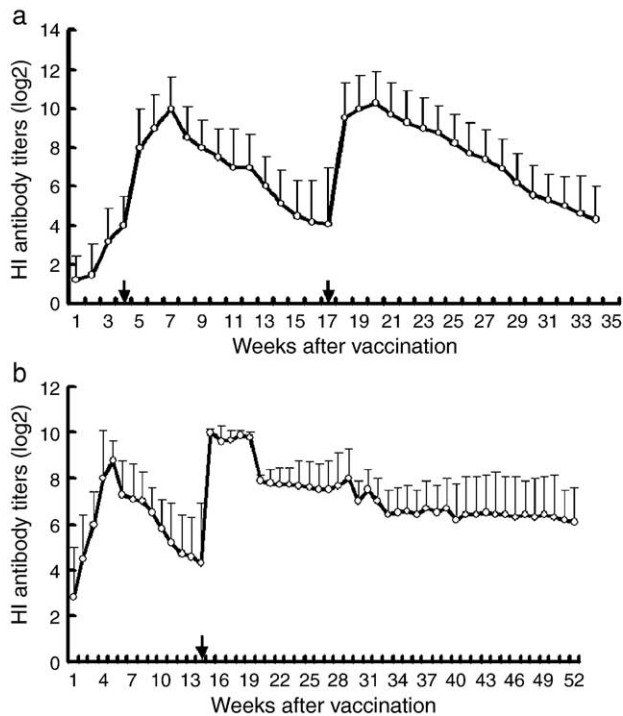


Fig. 3. HI antibody duration induced by inactivated vaccine derived from the Re-1 strain in geese (a) and ducks (b). Field geese were vaccinated 2 more times with 1.5 ml of the vaccine preparation with 13.8 μ g HA protein at 4 and 17 weeks, respectively, after the first shot with 0.5 ml of the vaccine preparation containing 4.6 μ g HA protein. Ducks received the second shot of 1.0 ml of the vaccine preparation with 9.2 μ g HA protein at 14 weeks after the first shot with 0.5 ml of the vaccine preparation containing 4.6 μ g HA protein. Sera were collected randomly from 20 of each type of birds on a weekly base for the HI antibody detection. The bars indicate the standard deviation, and the arrows indicate the time points for the second or third shots.

period, the geese were given 3 shots in total. Geese developed HI antibody very slowly after the first shot with 0.5 ml of the vaccine containing 4.6 μ g HA protein, and the titers reached 3log₂ by 3 weeks p.v and increased slightly at 4 weeks p.v. However, the antibody increased sharply and reached the peak of 10log₂ at 3 weeks after the second immunization with a high dosage of 1.5 ml vaccine containing 13.8 μ g HA protein and then gradually declined to 4log₂ by 13 weeks (17 weeks after the first immunization). The pattern of the antibody titers after the third shot is similar to that induced by the second shot, but the antibody duration was 4 weeks longer than the previous one. Virus was not detected from any swabs that were randomly collected from 20 geese every 2 weeks.

When the HI antibody declined to 4log₂ (17 weeks after the third shot), 10 geese were transferred to negative pressure isolates in the laboratory and challenged with 10^{7.5}EID₅₀ of highly pathogenic H5N1 avian influenza virus DKSH/2004. As shown in Table 2, all of the vaccinated geese were completely protected from the virus challenge, no virus shedding, no clinical disease signs, and no deaths were observed, while all of the unvaccinated control geese

shed viruses from both oropharynx and cloacae and died within 9 days after challenge (Table 2).

Vaccine efficacy in ducks

Laboratory studies

Three-week-old avian influenza serological negative ducks were immunized with 0.5 ml of the Re-1 vaccine preparations and were challenged 3 weeks after immunization with highly pathogenic avian influenza virus. As shown in Table 2, all the vaccinated ducks were completely protected and stayed healthy, and virus shedding was not detected from any ducks during the 1-month observation period. However, all of the control ducks shed different titers of virus at day 3 or 5 post-challenge, and 13 of 15 ducks died before day 6 (Table 2).

Field studies

To investigate the antibody kinetics induced by vaccination in ducks, antisera were collected randomly from 20 of the vaccinated ducks in the field on a weekly base for HI antibody detection. As shown in Fig. 3b, ducks developed HI antibody very quickly compared with geese. The average HI antibody titer of 3log₂ was detected 1 week p.v., reached the peak of 8log₂ at 4 weeks p.v and then gradually declined to 4log₂ by 14 weeks p.v. The antibody titers increased rapidly to 10log₂ 1 week after the second shot and remained at 6log₂ 38 weeks later (52 weeks after first dose, the end of the observation period) (Fig. 3b). Virus was not detected from any swabs that were randomly collected from the ducks.

At 38 weeks after the second shot, 10 ducks from the field were transferred to the laboratory and challenged with 10^{7.5}EID₅₀ of the H5N1 highly pathogenic virus DKSH/04 i.n., all of the vaccinated ducks were protected from clinical disease and death, though low titers of virus (from undiluted samples) were detected from the oropharyngeal swabs of 2 ducks on day 3 p.c. All of the ducks in the control group shed viruses through the oropharynx, and 3 shed viruses through cloacae on day 3 p.c. Virus was also detected from the oropharyngeal swabs of 5 ducks and cloacal swabs of 3 ducks on day 5, while no virus was detected from any ducks on day 7 p.c. (Table 2). Only one duck in the control group died during the 2-week observation period, indicating that adult ducks are much more resistant to the H5N1 avian influenza viruses compared with the young ducks.

Discussion

Inactivated whole virus vaccines have been used in several countries in an effort to control the outbreaks caused by highly pathogenic H5 and H7 viruses (Capua et al., 2003; Ellis et al., 2004). Using plasmid-based reverse genetics, we have generated the low-pathogenicity/high-growth H5N1

virus Re-1 strain, which is antigenically well-matched with the H5N1 highly pathogenic viruses found in China. The animal studies indicate that the H5N1 vaccine derived from the Re-1 strain is immunogenic and efficient in chickens, ducks and geese.

Numerous studies have confirmed the efficacy of the oil-emulsion inactivated vaccine for avian influenza in chickens and turkeys (Abraham et al., 1988; Capua et al., 2003, 2004; Ellis et al., 2004; Lee et al., 2004; Liu et al., 2003a; Stone, 1987; Swayne et al., 2001), and most of the efficacy evaluations have been based on a challenge study performed a few weeks after vaccination, when the HI antibody titers reach or are around their peak. Our previous studies had indicated that the vaccinated chickens could be completely protected from highly pathogenic avian influenza virus challenge when the HI antibody titers to the challenge virus equaled or were greater than $4\log_2$, a titer that can be easily induced by inactivated vaccine immunization. Therefore, a challenge study conducted 3 or 4 weeks after vaccination actually may not reflect how well a vaccine will work or how long the protective immunity will last in vaccinated birds in the field. The challenge study and HI antibody detection performed in the present study demonstrated that the protective immunity in SPF chickens induced by the Re-1 inactivated vaccine last for 10 months, which is 4 months longer than the duration induced by the H5N2 vaccine (from a strain of A/turkey/England/N-28/73) currently used in China (data not shown). HA protein in the Re-1 and H5N2 vaccines are 2.8 μg and 1.9 μg per dose (0.3 ml), respectively, and the higher HA protein content may be correlated with the longer protection in the vaccinated chickens.

An ideal vaccine candidate would induce cross protection against viruses from different antigenic groups within the subtype. As we have reported, there is no evidence of significant antigenic drift of the H5N1 viruses isolated from 1996–2002 in China (Chen et al., 2004). However, certain viruses isolated in 2004 are quite different from the GSGD/96 virus. The HA gene homologies of DKSH/04 and CKTJ/04 with GSGD/96 virus are 94.6% and 94.3%, respectively, and the HI titers of DKSH/04 and CKTJ/04 to the antisera of GSGD/96 are 4- and 8-fold lower, respectively, than the homologous titers (data not shown). The present studies show that immunization with the Re-1 vaccine could induce complete protection against homologous GSGD/96 challenge and against the heterologous virus DKSH/04 as well.

Free-range domestic waterfowl have the opportunity to contact with wild birds and also domestic poultry and animals. Therefore, they play an important role in transmission of virus from wild birds to other domestic poultry and other animals, including humans. China is heavily populated with free-range domestic waterfowl, and the H5N1 virus has circulated in healthy ducks in Southern China for several years (Chen et al., 2004; Guan et al., 2002; Webster et al., 2002). The present study and Sturm-

Ramirez's recent report (Sturm-Ramirez et al., 2004) indicate that certain recently isolated H5N1 viruses are lethal for ducks. Therefore, vaccination of domestic waterfowl to prevent H5N1 virus infection is crucial for effective disease control and eradication in animals and for public health as well. In the present study, we demonstrate that an oil-emulsion inactivated vaccine derived from the Re-1 strain is immunogenic in ducks and geese. One dose of 0.5 ml of the vaccine immunized ducks or geese was completely protected from highly pathogenic H5N1 virus challenge, but the duration of protective immunity was different. Two shots of the vaccine in ducks induced more than 52 weeks protection. The reaction of geese to the vaccine is quite different from ducks and chickens. The antibody titers rose slowly after the first shot, and the duration was relatively short even after the second and third shots, compared with the duration in ducks and chickens, suggesting that the vaccination program for different avian species should be carefully planned for field applications.

Avian influenza vaccine induced protection was both dose- and vaccine strain-dependent. Swayne et al. reported that the H5 avian influenza viruses have been shown to replicate in vaccinated but clinically normal chickens, and transmission of AIV might potentially occur within vaccinated flocks (Swayne et al., 2001). However, Ellis et al. reported that H5 vaccine can interrupt virus transmission in chickens in a field setting (Ellis et al., 2004). In the present study, the field tests were conducted in the free-range geese and ducks on a farm where a highly pathogenic H5N1 avian influenza outbreak had taken place in the beginning of 2004. The negative results of the virus isolation from the swabs of the vaccinated birds indicate that they were free from H5 avian influenza viruses infection. The negative isolation results of the swabs from the challenged vaccinated birds indicate that the present Re-1 vaccine could prevent chickens, ducks and geese from both affliction with H5N1 virus infection and efficient shedding of virus.

It is reported that the use of an inactivated influenza vaccine containing the marker NA permits differentiating infected from vaccinated animals (DIVA) (Capua et al., 2003; Lee et al., 2004; Liu et al., 2003a). A previous report (Liu et al., 2003b) and our avian influenza strain surveillance data have revealed that multiple HA and NA subtypes of avian influenza viruses exist in the domestic poultry in China, therefore, using NA as a marker to differentiate the infected from the vaccinated birds may not be applicable. Qiao et al. (2003) reported that a recombinant fowlpox virus containing the HA and NA genes from H5N1 viruses was able to provide protection against lethal H7N1 viruses challenge, indicating that the NA protein also has an important role in the vaccine efficacy. Therefore, in the present study, we chose to use both the HA and NA genes of the H5N1 virus to generate vaccine strain to match and provide a better protection to the prevalent H5N1 viruses. Applications of unvaccinated sentinel birds should be a

highly recommended option for detection of virus that may introduce into the vaccinated flocks.

In summary, we generated a high-growth H5N1 reassortant as an inactivated vaccine seed virus by plasmid-based reverse genetics and demonstrated that one dose of inactivated oil-emulsion vaccine could induce 10 months of protective immune response in chickens. Moreover, we first provided evidence that the oil-emulsion inactivated vaccine is indeed immunogenic and then proved the efficacy in domestic ducks and geese, which demonstrates that it is actually feasible to apply vaccines to protecting domestic waterfowl from H5N1 influenza virus infection. It is worthy to note that complete control and eradication of H5N1 highly pathogenic avian influenza viruses only be ultimately achieved by a combination of vaccination, improved biosecurity, extensive surveillance and an effective monitoring program. An effective vaccine is one crucial player in this multi-part scenario.

Materials and methods

Viruses and cells

GSGD/96 was the first H5N1 HPAIV isolated in China and has been characterized as previously reported (Chen et al., 2004; Xu et al., 1999). CKTJ/04 and DKSH/04 were isolated during the 2004 outbreaks. The viruses were propagated in the allantoic cavity of 10-day-old SPF chicken embryonated eggs and kept in a -70°C freezer before use for RNA extraction and challenge study. Plaque assay was performed in MDCK cells and with or without the addition of 0.5 μg of TPCK trypsin (Klenk et al., 1975). Virus rescue was carried out in *Vero* cells maintained in DMEM with 10% of FBS (Invitrogen Corp., Carlsbad, CA, USA), HEPERS (Invitrogen Corp., Carlsbad, CA, USA) and penicillin/streptomycin.

Plasmids

Twelve plasmids including the 8 vRNA of all the genome fragments and 4 mRNA expression plasmids of PB2, PB1, PA and NP of the PR8 virus were kindly provided by Drs. Brownlee and Fodor from Oxford University. The construction of these plasmids has been described in a previous report (Subbarao et al., 2003). An mRNA and vRNA bi-directional transcription plasmid pBD was constructed by inserting the *PoII* promoter and Ribozyme sequence fragment of the plasmid p*PoII-SapI-Rib* (a gift from Dr. Peter Palese, Mount Sinai School of Medicine, New York) into the *XbaI* site of pCI (Promega) in the sequence of the CMV-Rib-*PoII-SV40* poly A signal. The full-length HA gene and NA gene of GSGD/96 were amplified by RT-PCR and inserted into the *SapI* site of pBD. The amino acid sequence of the cleavage site of the HA RERRRKKR↓GLF was changed into RETRF↓GLF by PCR as described by Li et al. (1999).

Virus rescue

A monolayer of *Vero* cells in a 6-well plate was prepared, and transfection was conducted within 24 h of the cells being planted. 0.4 μg of each plasmid (HA and NA bi-directional plasmids, the remaining 6 gene transcription plasmids from PR8 and 4 protein expression plasmids from PR8) were added into 250 μl Opti-MEM (Invitrogen Corp., Carlsbad, CA, USA) and were mixed by vortex. Twelve microliter of the transfection reagent Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) was added into 250 μl Opti-MEM and mixed well gently. Five minutes later, the diluted reagent was mixed with the plasmid, and the mixture was kept at room temperature for 20–30 min. The cells were washed twice with 2 ml Opti-MEM, and then the DNA–transfection reagent mixtures were added directly to the cells. After 16-h incubation at 37°C in the CO_2 incubator, the medium was replaced with 2 ml fresh Opti-MEM with the addition of 0.5 μg of TPCK trypsin and was kept for two more days at 37°C . Then, the supernatant was inoculated into the allantoic cavity of 10-day-old embryonated SPF eggs. After 48-h incubation at 35°C , the allantoic fluid was harvested, and the virus was identified by hemagglutination assay using 0.5% chickens red blood cells.

Growth property analysis

10-day-old SPF embryonated eggs were inoculated with 100EID₅₀ of different viruses, and 10 eggs from each group were harvested and pooled at 24, 36, 48, 60 and 72 h, respectively, after inoculation. The HA titers were checked by using 0.5% chicken red blood cells.

Pathogenicity studies in chickens

Ten 4-week-old White Leghorn SPF chickens housed in isolator cages were inoculated with wt GSGD/96 and Re-1 transfectant viruses at a standard dose (0.2 ml of a 1:10 dilution of stock virus) by the intravenous (i.v.) route and were kept for 14 days for observation of disease signs and death. Oropharyngeal and cloacal swabs were collected for virus isolation on day 3 post-infection (p.i). On day 14 p.i., all surviving chickens were euthanized, and sera were collected and tested for evidence of seroconversion by agar gel precipitin (AGP) test using a baculovirus expressed nucleoprotein of influenza A virus as antigen and HI tests.

Preparation of formalin-inactivated vaccines

Virus was inoculated into the allantoic cavities of 10-day-old embryonated eggs and was harvested after 72-h incubation at 35°C . To detect the content of the HA protein, we run the SDS-PAGE of the freshly harvested allantoic fluid and scanned the gel using the GeneSnap software of Bio Imaging Systems (SYNGENE), and the

protein of the HA band (which has been confirmed by Western blotting analysis using the H5HA DNA vaccine immunized SPF chicken antisera in the preliminary analysis) was quantified with the GenTools software using the standard BSA protein. Then, the virus was inactivated by adding 0.2% formalin (v/v) and kept at 37 °C for 24 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of formalin-treated allantoic fluid in embryonated eggs. One part of the inactivated allantoic fluid was emulsified in 2 parts of paraffin oil (Hangzhou Oil Refining Company, Hangzhou, China) (volume/volume), which is currently used commercially as adjuvant for veterinary vaccine production. The HA protein content in the final vaccine preparation is 9.2 µg/ml.

Immunogenicity and efficacy of a formalin-inactivated Re-1 virus vaccine in chickens, geese and ducks

Chickens

Two groups of thirty-two 3-week-old white Leghorn SPF chickens were injected intramuscular (i.m.) with 0.3 ml PBS or formalin-inactivated vaccine preparations containing 2.8 µg HA antigen. Sera were collected randomly from 8 chickens of each group on a weekly base for HI antibody detection using the WHO standard method. Eight chickens from each group were challenged with 10^7 EID₅₀ of the homologous virus GSGD/96 intranasally at 2, 3 and 43 weeks, respectively, post-vaccination (p.v.). Oropharyngeal and cloacal swabs of the chickens were collected on days 3, 5 and 7 post-challenge (p.c.) for virus titration, and chickens were observed for disease signs and death for 2 weeks after challenge.

Another experiment was conducted to evaluate the protection against 2004 H5N1 isolates. Two groups of eight 3-week-old white leghorn chickens were vaccinated with 0.3 ml of the vaccine preparations and were challenged 3 weeks p.v. with 10^7 EID₅₀ of two H5N1 viruses, CKTJ/04 and DKSH/04, respectively. Oropharyngeal and cloacal swabs of chickens were collected on days 3, 5 and 7 post-challenge (p.c.) for virus isolation, and chickens were observed for disease signs and death for 2 weeks after challenge.

Geese

Laboratory studies. Groups of ten 3-week-old avian influenza serological negative geese (local strain) were injected intramuscularly (i. m.) with 0.5 ml PBS or Re-1 vaccine preparations (containing 4.6 µg HA antigen), and five geese from each group were challenged with $10^{7.5}$ EID₅₀ of DKSH/04, at 2 or 3 weeks after vaccination. Oropharyngeal and cloacal swabs were collected on days 3, 5 and 7 post-challenge (p.c.) for virus isolation, and geese were observed for disease signs and death for 2 weeks p.c.

Field studies. We conducted the test on a small-scale farm to learn the duration of the protective antibody and efficacy induced by the inactivated vaccine in the geese. The geese were serologically confirmed as avian influenza negative by AGP test. Two hundred 2-week-old geese were vaccinated with 0.5 ml of the vaccine preparations containing 4.6 µg of HA antigen, and the second and third shots were applied with a high dosage of 1.5 ml of the vaccine preparations containing 13.8 µg of the HA protein 4 weeks and 17 weeks, respectively, after the first shot, when the HI antibody titers reduced to 4log₂. Sera were collected randomly from 20 geese on a weekly base for HI antibody detection. Every 2 weeks after vaccination, oropharyngeal and cloacal swabs were collected randomly from 20 of each type of birds for virus isolation. At the end of observation periods, 10 geese were shipped to the laboratory for the challenge study, as described above. The same age avian influenza negative birds confirmed by AGP test were used as control.

Ducks

Laboratory studies. Avian influenza serological negative ducks were used. Groups of ten 3-week-old ducks (a local outbred strain of sheldrake) were injected intramuscular (i.m.) with 0.5 ml of the vaccine preparations (containing 4.6 µg HA antigen), another group of five ducks were unvaccinated as a control. Ducks were challenged with $10^{7.5}$ EID₅₀ DKSH/04 3 weeks p.v. Oropharyngeal and cloacal swabs were collected on days 3, 5, 7, 10, 20 and 30 days post-challenge (p.c.) for virus titration. Ducks were observed for disease signs and death for 30 days after challenge. The duck experiments were repeated two more times independently.

Field studies. Ducks in a small-scale farm were serologically confirmed as avian influenza negative by the AGP test. Four hundred 4-week-old ducks were injected i.m. with the vaccine preparations. Sera were collected randomly from 20 ducks on a weekly base for HI antibody detection. The second shot of 1 ml vaccine preparation containing 9.2 µg HA protein was applied at 14 weeks after the first shot when the average HI antibody titer declined to 4log₂. Every 2 weeks after vaccination, oropharyngeal and cloacal swabs were collected randomly from 20 of birds for virus isolation. At the end of observation periods, 10 ducks were shipped to the laboratory for the challenge study. The same age avian influenza negative birds were used as control.

Serologic tests and virus titration. Hemagglutination inhibition assays were performed by following the WHO standard. Each swab was washed in 1 ml cold PBS, and virus titration was conducted in 10-day-old SPF embryonated chickens' eggs and calculated by the method of Reed and Muench (1938).

Laboratory facility. All experiments related to the HPAIV were conducted in a P3 facility.

Acknowledgments

We gratefully acknowledge Dr. Peter Palese for providing the plasmid *pPoll-SapI-ribozyme*, Drs. Nancy Cox and Kanta Subbarao for providing the plasmid *pBD* and Drs. George Brownlee and Ervin Fodor for providing the PR8 plasmids. This study was supported by Chinese Science and Technology Development Program (863) 2004AA3071, Chinese National S&T Plan Grant 2004BA519A03, Shanghai Agricultural S&T Plan Grant 2004121 and Chinese National Key Basic Research Program (973) G199901190.

References

- Abraham, A., Sivanandan, V., Karunakaran, D., Halvorson, D.A., Newman, J.A., 1988. Comparative serological evaluation of avian influenza vaccine in turkeys. *Avian Dis.* 32, 659–662.
- Alexander, D.J., Parsons, G., Manvell, R.J., 1986. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol.* 15, 647–662.
- Anonymous, 2003. Avian influenza virus reappears in Hong Kong special administrative region. *Bull. W.H.O.* 81, 232.
- Capua, I., Terregino, C., Cattoli, G., Mutinelli, F., Rodriguez, J.F., 2003. Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. *Avian Pathol.* 32, 47–55.
- Capua, I., Terregino, C., Cattoli, G., Toffan, A., 2004. Increased resistance of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus. *Avian Pathol.* 33, 158–163.
- Chen, H., Yu, K., Jiang, Y., Tang, X., 2001. DNA immunization elicits high HI antibody and protects chickens from AIV challenge. *Int. Congr. Ser.* 1219, 917–921.
- Chen, H., Subbarao, K., Swayne, D., Chen, Q., Lu, X., Katz, J., Cox, N., Matsuoka, Y., 2003. Generation and evaluation of a high-growth reassortant H9N2 influenza A virus as a pandemic vaccine candidate. *Vaccine* 21, 1974–1979.
- Chen, H., Deng, G., Li, Z., Tian, G., Li, Y., Jiao, P., Zhang, L., Liu, Z., Webster, R.G., Yu, K., 2004. The evolution of H5N1 influenza viruses in ducks in southern China. *Proc. Natl. Acad. Sci. U.S.A.* 101, 10452–10457.
- Chin, P.S., Hoffmann, E., Webby, R., Webster, R.G., Guan, Y., Peiris, M., Shortridge, K.F., 2002. Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. *J. Virol.* 76, 507–516.
- Claas, E.C., Osterhaus, A.D., van Beek, R., De Jong, J.C., Rimmelzwaan, G.F., Senne, D.A., Krauss, S., Shortridge, K.F., Webster, R.G., 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. *Lancet* 351, 472–477.
- Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L.A., Wong, W., Guan, Y., Peiris, M., 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. *Avian Pathol.* 33, 405–412.
- Fodor, E., Devenish, L., Engelhardt, O.G., Palese, P., Brownlee, G.G., Garcia-Sastre, A., 1999. Rescue of influenza A virus from recombinant DNA. *J. Virol.* 73, 9679–9682.
- Garcia-Sastre, A., Palese, P., 1993. Genetic manipulation of negative-strand RNA virus genomes. *Annu. Rev. Microbiol.* 47, 765–790 (Review).
- Guan, Y., Peiris, J.S.M., Lipatov, A.S., Ellis, T.M., Dyrting, K.C., Krauss, S., Zhang, L.J., Webster, R.G., Shortridge, K.F., 2002. Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. *Proc. Natl. Acad. Sci. U.S.A.* 99, 8950–8955.
- Hatta, M., Gao, P., Halfmann, P., Kawaoka, Y., 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293, 1840–1842.
- Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., Webster, R.G., 2000a. A DNA transfection system for generation of influenza A virus from eight plasmids. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6108–6113.
- Hoffmann, E., Stech, J., Leneva, I., Krauss, S., Scholtissek, C., Chin, P.S., Peiris, M., Shortridge, K.F., Webster, R.G., 2000b. Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1? *J. Virol.* 74, 6309–6315.
- Hoffmann, E., Krauss, S., Perez, D., Webby, R., Webster, R.G., 2002. Eight-plasmid system for rapid generation of influenza virus vaccines. *Vaccine* 20, 3165–3170.
- Karunakaran, D., Newman, J.A., Halvorson, D.A., Abraham, A., 1987. Evaluation of inactivated influenza vaccines in market turkeys. *Avian Dis.* 31, 498–503.
- Keawcharoen, J., Oraveerakul, K., Kuiken, T., Fouchier, R.A., Amonsin, A., Payungporn, S., Noppornpanth, S., Wattanadorn, S., Theambooniers, A., Tantilertcharoen, R., Pattanarangsarn, R., Arya, N., Ratanakorn, P., Osterhaus, D.M., Poovorawan, Y., 2004. Avian influenza H5N1 in tigers and leopards. *Emerg. Infect. Dis.* 10, 2189–2191.
- Kilbourne, E.D., 1969. Future influenza vaccines and the use of genetic recombinants. *Bull. W.H.O.* 41, 643–645.
- Klenk, H.D., Rott, R., Orlich, M., Blodorn, J., 1975. Activation of influenza A viruses by trypsin treatment. *Virology* 68, 426–439.
- Lee, C.W., Senne, D.A., Suarez, D.L., 2004. Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (Differentiating Infected from Vaccinated Animals) strategy for the control of avian influenza. *Vaccine* 22, 3175–3181.
- Lee, C.W., Suarez, D.L., Tumpey, T.M., Sung, H.W., Kwon, Y.K., Lee, Y.J., Choi, J.G., Joh, S.J., Kim, M.C., Lee, E.K., Park, J.M., Lu, X., Katz, J.M., Spackman, E., Swayne, D.E., Kim, J.H., 2005. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J. Virol.* 79, 3692–3702.
- Li, S., Liu, C., Klimov, A., Subbarao, K., Perdue, M.L., Mo, D., Ji, Y., Woods, L., Hietala, S., Bryant, M., 1999. Recombinant influenza A virus vaccines for the pathogenic human A/Hong Kong/97 (H5N1) viruses. *J. Infect. Dis.* 179, 1132–1138.
- Lipatov, A.S., Webby, R.J., Govorkova, E.A., Krauss, S., Webster, R.G., 2005. Efficacy of H5 influenza vaccines produced by reverse genetics in a lethal mouse model. *J. Infect. Dis.* 191, 1216–1220.
- Liu, M., Wood, J.M., Ellis, T., Krauss, S., Seiler, P., Johnson, C., Hoffmann, E., Humbert, J., Hulse, D., Zhang, Y., Webster, R.G., Perez, D.R., 2003a. Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. *Virology* 314, 580–590.
- Liu, M., He, S., Walker, D., Zhou, N., Perez, D.R., Mo, B., Li, F., Huang, X., Webster, R.G., Webby, R.J., 2003b. The influenza virus gene pool in a poultry market in South Central China. *Virology* 305, 267–275.
- Marsh, G.A., Tannock, G.A., 2005. The role of reverse genetics in the development of vaccines against respiratory viruses. *Expert Opin. Biol. Ther.* 5, 369–380.
- Mase, M., Tsukamoto, K., Imada, T., Imai, K., Tanimura, N., Nakamura, K., Yamamoto, Y., Hitomi, T., Kira, T., Nakai, T., Kiso, M., Horimoto, T., Kawaoka, Y., Yamaguchi, S., 2005. Characterization of H5N1 influenza A viruses isolated during the 2003–2004 influenza outbreaks in Japan. *Virology* 332, 167–176.
- Neumann, G., Watanabe, T., Ito, H., Watanabe, S., Goto, H., Gao, P., Hughes, M., Perez, D.R., Donis, R., Hoffmann, E., Hobom, G., Kawaoka, Y., 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9345–9350.

- Nguyen, D.C., Uyeki, T.M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H., Xu, X., Balish, A., Klimov, A., Tumpey, T.M., Swayne, D.E., Huynh, L.P., Nghiem, H.K., Nguyen, H.H., Hoang, L.T., Cox, N.J., Katz, J.M., 2005. Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J. Virol.* 79, 4201–4212.
- Nicolson, C., Major, D., Wood, J.M., Robertson, J.S., 2005. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. *Vaccine* 23, 2943–2952.
- Peiris, J.S., Yu, W.C., Leung, C.W., Cheung, C.Y., Ng, W.F., Nicholls, J.M., Ng, T.K., Chan, K.H., Lai, S.T., Lim, W.L., Yuen, K.Y., Guan, Y., 2004. Re-emergence of fatal human influenza A subtype H5N1 disease. *Lancet* 363, 617–619.
- Perdue, M.L., Garcia, M., Senne, D.A., Fraire, M., 1997. Virulence associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. *Virus Res.* 49, 173–186.
- Perkins, L.E., Swayne, D.E., 2002. Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Dis.* 46, 53–63.
- Puthavathana, P., Auewarakul, P., Charoenying, P.C., Sangsiriwut, K., Pooruk, P., Boonnak, K., Khanyok, R., Thawachsupha, P., Kijphati, R., Sawanpanyalert, P., 2005. Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. *J. Gen. Virol.* 86, 423–433.
- Qiao, C.L., Yu, K.Z., Jiang, Y.P., Jia, Y.Q., Tian, G.B., Liu, M., Deng, G.H., Wang, X.R., Meng, Q.W., Tang, X.Y., 2003. Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. *Avian Pathol.* 32, 25–32.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Richmond, J.Y., McKinney, R.W., 1993. "Biosafety in Microbiological and Biomedical Laboratories." U.S. Department of Health and Human Services, CDC/NIH, Washington, D.C.
- Schickli, J.H., Flandorfer, A., Nakaya, T., Martinez-Sobrido, L., Garcia-Sastre, A., Palese, P., 2001. Plasmid-only rescue of influenza A virus vaccine candidates. *Philos. Trans. R. Soc. Lond., B Biol. Sci.* 356, 1965–1973.
- Senne, D.A., Panigrahy, B., Kawaoka, Y., Pearson, J.E., Suss, J., Lipkind, M., Kida, H., Webster, R.G., 1996. Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. *Avian Dis.* 40, 425–437.
- Shortridge, K.F., Zhou, N.N., Guan, Y., Gao, P., Ito, T., Kawaoka, Y., Kodihalli, S., Krauss, S., Markwell, D., Murti, K.G., Norwood, M., Senne, D., Sims, L., Takada, A., Webster, R.G., 1998. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* 252, 331–342.
- Sims, L.D., Guan, Y., Ellis, T.M., Liu, K.K., Dyrting, K., Wong, H., Kung, N.Y., Shortridge, K.F., Peiris, M., 2003. An update on avian influenza in Hong Kong 2002. *Avian Dis.* 47, 1083–1086 (Suppl.).
- Stone, H.D., 1987. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. *Avian Dis.* 31, 483–490.
- Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., Poon, L., Guan, Y., Peiris, M., Webster, R.G., 2004. Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *J. Virol.* 78, 4892–4901.
- Subbarao, K., Katz, J.M., 2004. Influenza vaccines generated by reverse genetics. *Curr. Top. Microbiol. Immunol.* 283, 313–342.
- Subbarao, K., Klimov, A., Katz, J., Regnery, H., Lim, W., Hall, H., Perdue, M., Swayne, D., Bender, C., Huang, J., Hemphill, M., Rowe, T., Shaw, M., Xu, X., Fukuda, K., Cox, N., 1998. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. *Science* 279, 393–396.
- Subbarao, K., Chen, H., Swayne, D., Mingay, L., Fodor, E., Brownlee, G., Xu, X., Lu, X., Katz, J., Cox, N., Matsuoka, Y., 2003. Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. *Virology* 305, 192–200.
- Swayne, D.E., Beck, J.R., Perdue, M.L., Beard, C.W., 2001. Efficacy of vaccines in chickens against highly pathogenic Hong Kong H5N1 avian influenza. *Avian Dis.* 45, 355–365.
- Takada, A., Kuboki, N., Okazaki, K., Ninomiya, A., Tanaka, H., Ozaki, H., Itamura, S., Nishimura, H., Enami, M., Tashiro, M., Shortridge, K.F., Kida, H., 1999. Avirulent avian influenza virus as a vaccine strain against a potential human pandemic. *J. Virol.* 73, 8303–8307.
- Thanawongnuwech, R., 2005. Probable tiger-to-tiger transmission of avian influenza H5N1. *Emerg. Infect. Dis.* 11, 699–701.
- Tran, T.H., Nguyen, T.D., Nguyen, T.L., Luong, T.S., Pham, P.M., Nguyen, V.C., Pham, T.S., Vo, C.D., Le, T.Q., Ngo, T.T., Dao, B.K., Le, P.P., Nguyen, T.T., Hoang, T.L., Cao, V.T., Le, T.G., Nguyen, D.T., Le, H.N., Nguyen, K.T., Le, H.S., Le, V.T., Christiane, D., Tran, T., Menno, T., de, J., Schultsz, C., Cheng, P., Lim, W., Horby, P., Farrar, J., World Health Organization International Avian Influenza Investigative Team, 2004. Avian influenza A (H5N1) in 10 patients in Vietnam. *N. Engl. J. Med.* 350, 1179–1188.
- Webby, R.J., Perez, D.R., Coleman, J.S., Guan, Y., Knight, J.H., Govorkova, E.A., McClain-Moss, L.R., Peiris, J.S., Rehg, J.E., Tuomanen, E.I., Webster, R.G., 2004. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. *Lancet* 363, 1099–1103.
- Webster, R.G., Guan, Y., Peiris, M., Walker, D., Krauss, S., Zhou, N.N., Govorkova, E.A., Ellis, T.M., Dyrting, K.C., Sit, T., Perez, D.R., Shortridge, K.F., 2002. Characterization of H5N1 influenza viruses that continue to circulate in geese in southeastern China. *J. Virol.* 76, 118–126.
- Xu, X.Y., Subbarao, K., Cox, N.J., Guo, Y.J., 1999. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 261, 15–19.
- Zambon, M., 1998. Laboratory containment for influenza A H5N1 viruses: level 2, level 3, or level 3? *Commun. Dis. Public Health* 1, 71–72.