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FULL PAPER

# A vaccine prepared from a non-pathogenic H5N1 influenza virus strain from the influenza virus library conferred protective immunity to chickens against the challenge with antigenically drifted highly pathogenic avian influenza virus

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## Abstract

**Inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library conferred protective immunity to chickens against the challenge of antigenically drifted highly pathogenic avian influenza virus (HPAIV), A/whooper swan/Hokkaido/1/2008 (H5N1). The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-A/whooper swan/Mongolia/3/2005 (H5N1), which is more antigenically related to the challenge virus strain, in chickens.**

Keywords: *Antigenically drifted HPAIV, vaccine*

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## Introduction

H5N1 highly pathogenic avian influenza virus (HPAIV) is causing panzootic outbreaks in poultry in Eurasia and Africa, posing serious concern for public health as well as live stock industry. The outbreaks of highly pathogenic avian influenza caused by H5N1 viruses that spread to 62 countries have taken toll of 300 million poultry (dead or been killed) and 15 countries with human fatalities[5]. In addition, the H5N1 HPAIVs had returned to migratory birds, spread to Eurasia and Africa[2,6]. Since each of the hemagglutinin (HA) genes of pandemic influenza viruses has been originated from avian influenza viruses[14], is now a potential pandemic threat. H5N1 viruses isolated from water birds found dead in Mongolia on the way back to their nesting lakes in Siberia in April to May in 2005, 2006, 2009 and 2010 were genetically closely related to H5N1 viruses isolated from birds in China, Iraq, Croatia, Nigeria, Korea and Japan[2,6,10]. Viruses similar to those have spread world-wide and it is therefore, a serious concern that these HPAIVs persists in Eurasia may perpetuate in the lakes where they nest in summer and that those birds may bring HPAIVs to the south in autumn.

Stamping-out and movement restriction are the standard measures for the control of highly pathogenic avian influenza (HPAI) in poultry and found to be successful in rapid eradication of the HPAIV infection[4]. Vaccination is a limited application as an optional tool when stamping-out is not effective enough to control the disease[1]. Vaccination may be an optional measure in cases where the disease spread widely. Many commercial vaccines have been prepared from viruses of the North American lineage. These vaccines may be less effective for the control of current HPAI outbreaks caused by the infection with viruses of the Eurasian lineage in Asia[5]. Inactivated influenza vaccines for the control of the circulating avian influenza particularly in Asia, therefore, should be prepared from an H5N1 virus strain belonging to the Eurasian lineage.

The OIE Reference Laboratory for HPAI at Hokkaido University has established the library of influenza viruses of all HA and neuraminidase (NA) subtypes and their genes[6]. The previous study[12] has demonstrated that the library of a panel of influenza virus strains isolated from natural hosts is useful for the preparedness for future pandemics. These influenza virus strains are stored in the library and have been used for the purpose of vaccine production and diagnosis.

Prolonged endemics of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries[9]. Previous study[5,8], showed that avian influenza vaccine prepared from non-pathogenic avian influenza viruses from the library conferred protective immunity against the challenge virus of antigenically similar. Ideally, vaccine strains that are antigenically and genetically closely related to the circulating variant strain and induce immunity against antigenically drifted virus are preferable. In the present study, the efficacy of the vaccine prepared from a non-pathogenic influenza virus strain A/duck/Hokkaido/Vac-1/2004 (H5N1) from the virus library was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05 (H5N1) by reverse genetics against the challenge with antigenically drifted HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) in chickens.

## Materials and Methods

*Viruses:* Influenza viruses, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) and A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05) and mutant A/whooper swan/Mongolia/

3/2005 (H5N1) (ΔRRRRK rg-Mon/05), of clade 2.3.2, classical and clade 2.2 respectively were used. All viruses used in this study have been propagated and characterized antigenically and genetically as described[9]. All viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 hrs and stored at −80°C until use.

Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were used for vaccine preparation. A non-pathogenic avian influenza Dk/Vac-1/04 virus from the library, was generated as a reassortant virus between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1)[5]. Ws/Mon/05 virus isolated from a whooper swan (*Cygnus Cygnus*) found dead in Lake Khunt nuur, Mongolia[10] was genetically modified by reverse genetics with site-directed mutagenesis to generate a mutant ΔRRRRK rg-Mon/05 strain.

Meanwhile, Ws/Hok/08 (H5N1) virus that was isolated from whooper swan found dead at Notsuke Peninsular, in Hokkaido, Japan in May on their way back to their nesting lakes in northern territories[9] was used as the challenge virus.

*Preparation of a genetically modified H5N1 HPAIV:* Ws/Mon/05 was genetically modified by reverse genetics with site-directed mutagenesis as described[7]. Briefly, the amino acid RRRRK at the cleavage site of the HA were deleted and replaced with amino acid T by site-directed mutagenesis. The T mutation was introduced into the HA of Ws/Mon/05 cloned into the pHW2000 expression vector, using a Quick Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The presence of the desired mutations was confirmed by sequencing the full length of the cloned HA genes. The mutant virus was designated ΔRRRRK rg-Mon/05 and confirmed to be apathogenic to chickens.

*Intravenous pathogenicity (IVPI) of ΔRRRRK rg-Mon/05 in chickens:* The intravenous pathogenicity index of ΔRRRRK rg-Mon/05 was carried out according to the OIE standard method[8]. Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 6-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo) at a BSL 3 facility at the Hokkaido University Research Center for Zoonosis Control, Japan. All animal experiments were conducted in accordance to guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

*Vaccine preparation:* A virus suspension of Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 were inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4°C respectively. Virus inactivation was confirmed by inoculation into embryonated chicken eggs. The inactivated Dk/Vac-1/04 and ΔRRRRK rg-Mon/05 viruses suspension were diluted with phosphate-buffered saline (pH = 7.2) (PBS) to appropriate concentrations based on HA titers and adjuvanted as described[5,11].

*Potency test of vaccine efficacy in chickens against antigenically drifted strains Ws/Hok/08:* The potency of the vaccines was evaluated by challenging chickens inoculated with vaccines prepared from Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 with antigenically drifted Ws/Hok/08. Thirty 4-week-old chickens were divided into three groups and the inactivated avian influenza virus Dk/Vac-1/04 or ΔRRRRK rg-Mon/05 vaccines were intramuscularly inoculated to ten chickens respectively as described[5,11]. PBS was inoculated in ten control chickens. Three weeks after vaccination, all chickens were challenged intranasally with a dose $10^3$ 50% chicken lethal dose ($CLD_{50}$) of Ws/Hok/08. Four chickens of each group were sacrificed on day 3 post-challenge and the remaining six chickens were observed

clinically for 14 days. When chickens died or were sacrified, tracheal and cloacal swabs and their tissues (trachea, kidney, colon) were collected. Virus infectivity titers were determined by plaque assays.

*Plaque assays:* Virus infectivity titers in swabs and tissue samples were determined by plaque assay as described[13]. Briefly, MDCK cells were grown in 6-well tissue culture plates, when monolayer became confluent, ten-fold serial dilutions of swab and tissue samples were prepared in minimal essential medium (MEM) and 200 μl of each dilution was added to each well. After 1 hour adsorption at 37°C the inoculums were removed and the cells were overlaid with Eagle's MEM containing 0.9% Bacto-Agar (Difco). After incubation for 24–48 hrs, second overlay containing neutral red (0.005%) was made and plaques were enumerated after overnight incubation. The limit of virus detection was $10^3$ plaque forming units (PFU)/g of tissues or ml of swabs.

## Results

*Antigenic relatedness among the challenge and vaccine strain viruses*

Okamatsu *et al.*, 2010[9] has shown that

**Table 1. HI titers of the sera of chickens before (0) and 14 days after challenge**

| Vaccine strains | #[a] | HI titers with the following antigens on the day post challenge | | | | | |
|---|---|---|---|---|---|---|---|
| | | Dk/Vac-1/04 | | Ws/Mon/05 | | Ws/Hok/08 | |
| | | 0 | 14 | 0 | 14 | 0 | 14 |
| Vac-1/04 | 1 | 640 | -[b] | 320 | - | 320 | - |
| | 2 | 1280 | - | 160 | - | 160 | - |
| | 3 | 1280 | - | 160 | - | 80 | - |
| | 4 | 5120 | - | 1280 | - | 80 | - |
| | 5 | 5120 | 10240 | 1280 | 5120 | 80 | 640 |
| | 6 | 640 | 2560 | 160 | 1280 | 160 | 320 |
| | 7 | 2560 | 20480 | 160 | 5120 | 40 | 2560 |
| | 8 | 640 | 2560 | 160 | 1280 | 80 | 640 |
| | 9 | 2560 | 5120 | 80 | 1280 | 40 | 640 |
| | 10 | 5120 | 20480 | 320 | 640 | 40 | 160 |
| rg-Mon/05 | 11 | 40 | - | 80 | - | 80 | - |
| | 12 | 20 | - | 40 | - | 80 | - |
| | 13 | 80 | - | 640 | - | 320 | - |
| | 14 | 40 | - | 320 | - | 160 | - |
| | 15 | 320 | 1280 | 640 | 2560 | 640 | 1280 |
| | 16 | 1280 | 2560 | 1280 | 2560 | 160 | 640 |
| | 17 | 640 | 640 | 640 | 640 | 80 | 160 |
| | 18 | 1280 | 2560 | 1280 | 5120 | 320 | 1280 |
| | 19 | 640 | 1280 | 640 | 1280 | 320 | 640 |
| | 20 | 320 | 1280 | 640 | 1280 | 320 | 1280 |

[a]: Chicken number
[b]: no samples tested

antigenicities of the HA of the isolates in 2008 were different from the H5N1 viruses isolated from wild birds and poultry before 2007. These findings indicate that the challenge virus strain Ws/Hok/08 is antigenically different from Dk/Vac-1/04.

*Pathogenicity of ΔRRRRK rg-Mon/05 (H5N1)*

None of the chickens inoculated intravenously with ΔRRRRK rg-Mon/05 showed clinical signs in the 10 days observation period. The IVPI value of the strain is 0.00 indicating that ΔRRRRK rg-Mon/05 is non-apathogenic for chickens. No virus was recovered from organs of the chickens inoculated with ΔRRRRK rg-Mon/05 except one bird from which organ sample trachea showed $10^{1.75}$ $EID_{50}$/g.

*Potency of vaccines in chickens*

Table 1 shows serum HI antibody titers in the vaccinated chickens. The HI antibody titers of the vaccinated chickens showed increase in the antibody titers after 3 weeks of vaccination. Chickens inoculated with the test vaccines were challenged with HPAIV, Ws/Hok/08 on 3 weeks post vaccination. Two weeks after challenge, approximately $2^4$ fold of HI antibody titers were increased in all vaccinated chickens to the homologous and heterologus viruses indicating virus replication occurred. In addition, all vaccinated chickens survived after challenge throughout 14 observation days without showing any disease signs, whereas all of the control chickens died within 2 days post challenge (Fig. 1). Viruses were not recovered from swabs and tissue samples of any of the vaccinated chickens after challenge by plaque assay.

## Discussion

It is strongly recommended that stamping-out without misuse of vaccine is the best option for the eradication of HPAIV. Vaccination may be an optional tool in cases where the infection spreads widely[1]. Such a vaccine should ideally meet the following criteria: (i) safe for both hosts and the environment; (ii) economically feasible; and (iii) efficacious.

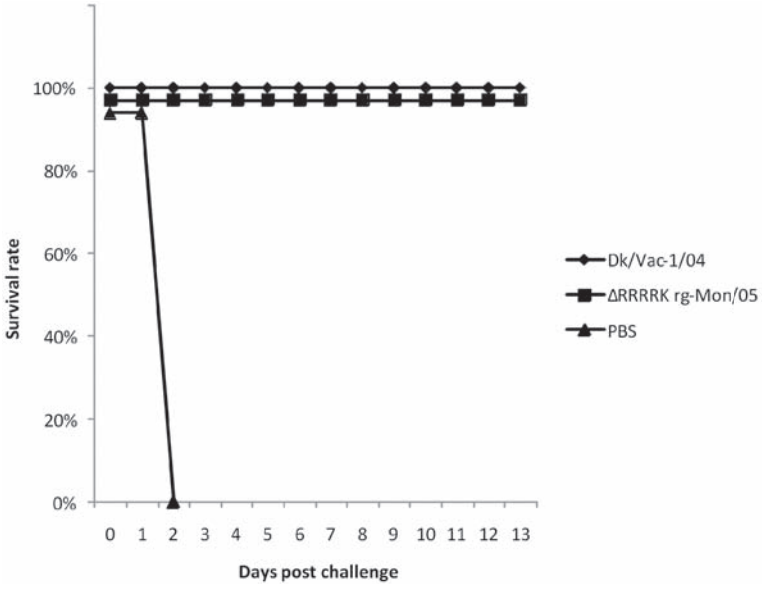


**Fig. 1. Survival of chickens after challenge with Ws/Hok/08.** Ten 4-week-old chickens in each group were vaccinated with Dk/Vac-1/04, and ΔRRRRK rg-Mon/05 respectively. Three weeks after vaccination, chickens were challenged with Ws/Hok/08 (H5N1) virus. Control chickens (black triangle) were dead within 24 to 48 hr after challenge. Vaccinated chickens were observed for 14 days.

In the present study, both vaccine strains induced sufficient antibody response against the challenge with phylogenetically and antigenically different HPAIV. Previous studies[5,9] have shown that Dk/Vac-1/04 vaccine protect chickens from clinical signs and induced antibodies against homologous and heterologous strains after challenge. In the present study, it was shown that vaccinated chickens with Dk/Vac-1/04 not only induced antibodies against homologous and heterologous strain but conferred protective immunity to chickens against the challenge of antigenically drifted HPAIV. These findings indicate that vaccine prepared from non-pathogenic avian influenza virus from the virus library is efficacious and protect chickens from HPAI. Furthermore, chickens even challenged with higher doses of viral challenge ($10^3$ $CLD_{50}$) did not show clinical signs indicating that the vaccines induced sufficient protective immunity in chickens to prevent clinical manifestations.

On the basis of the findings in the present study, inactivated influenza virus vaccine prepared from a non-pathogenic influenza virus strain from the virus library conferred protective immunity against the challenge with antigenically drifted HPAIV. The efficacy of the vaccine was comparable to that prepared from genetically modified HPAIV strain ΔRRRRK rg-Mon/05, which is more antigenically related to the challenge virus strain, in chickens. Therefore, it is proposed that vaccine strain selected from the non-pathogenic influenza virus library is efficacious and safe in protecting chickens from HPAI.

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