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Title	Development of vaccine strains of H5 and H7 influenza viruses
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Citation	Japanese Journal of Veterinary Research, 55(2-3), 93-98
Issue Date	2008-02-09
DOI	10.14943/jjvr.55.2-3.93
Doc URL	http://hdl.handle.net/2115/32369
Type	bulletin (article)
File Information	55(2-3)_93-98.pdf

Instructions for use

Development of vaccine strains of H5 and H7 influenza viruses

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Abstract

To establish vaccine strains of H5 and H7 influenza viruses, A/duck/Hokkaido/Vac-1/04 (H5N1) [Vac-1/04 (H5N1)], A/duck/Hokkaido/Vac-3/07 (H5N1) [Vac-3/07 (H5N1)], and A/duck/Hokkaido/ Vac-2/04 (H7N7) [Vac-2/04 (H7N7)] were generated from non-pathogenic avian influenza viruses isolated from migratory ducks. Vac-1/04 (H5N1) and Vac-3/07 (H5N1) were generated by genetic reassortment between H5N2 or H5N3 virus as an HA gene provider and H7N1 or H6N1 viruses as an NA gene provider. Vac-2/04 (H7N7) was a genetic reassortant obtained using H7N7 and H9 N2 viruses to give high growth character of the H9N2 virus in chicken embryonated eggs. The results of sequence analyses and experimental infections revealed that these H5N1 and H7N7 reassortant viruses were non-pathogenic in chickens and embryos, and had good growth potential in embryonated eggs. These viruses should be useful to develop vaccines against H5 and H7 highly pathogenic avian influenza viruses.

Key Words: genetic reassortant, H5, H7, influenza, vaccine

Introduction

Highly pathogenic avian influenza (HPAI) caused by H5 and H7 influenza viruses have occurred worldwide, leading to serious economic losses in poultry industries. In Japan, there had been no outbreak since 1925 when HPAI caused by a H7N7 virus occurred in Chiba prefecture[13). In the beginning of 2004, HPAI outbreaks caused by H5N1 viruses occurred at chicken farms in

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Yamaguchi and Kyoto prefectures[7,10]. H5N1 infections then reoccurred on poultry farms in Miyazaki and Okayama prefectures in 2007. The depopulation of chickens in the relevant farms and appropriate control measures successfully prevented the spread of HPAI.

Standard countermeasures for the control of HPAI outbreaks are testing and culling of affected chickens. However, when outbreaks spread to a broad area, being uncontrollable, ring vaccination would be an optional measure to reduce the concentration of the virus and, hence, suppress its spread[3]. In response to the 2004 outbreaks in Japan, commercial vaccines were urgently imported from Mexico as stockpiles. However, these vaccines have been suspected that it might be inadequate to control current outbreaks in Asia because they have been produced from H5N2 viruses of North American lineage, which are antigenically different from those of Eurasian lineage. A domestic vaccine which has a similar antigenicity to viruses circulating in Asia at present has been awaited for stable production and provision in the case of a nation-wide outbreak which cannot be controlled by stamping out strategy.

The aim of the present study is to establish proper seed strains for vaccine manufacturing against the H5 and H7 influenza viruses in Asia. Here, we report the characteristics of non-pathogenic H5N1 and H7N7 viruses generated from parental viruses isolated from ducks, a natural host of influenza A viruses, by genetic reassortment.

Materials and Methods

Viruses: All the parental viruses for generating vaccine candidate strains in this study were isolated in our laboratory from fecal samples of migratory ducks in Mongolia and Japan[8]. The viruses were propagated in ten-day-old embryonated chicken eggs for 48 hours at 35℃ and infectious allantoic fluids were used for generating of reassortant viruses.

Generation of genetic reassortant virus: To generate the H5N1 and H7N7 reassortant viruses, parental viruses were mixed and inoculated into the allantoic cavity of embryonated chicken eggs. After incubation at 35℃ for 24h or 48h, the allantoic fluids were collected, mixed with chicken antisera (α-A/Duck/Hong Kong/301/78 (H7N2) for A/duck/Hokkaido/Vac-1/04 (H5N1), α-A/turkey/England/73 (H7N3) and α-A/turkey/Massachusetts/3740/65 (H6N2) for A/duck/Hokkaido/Vac-3/07 (H5N1), and α-A/duck/Hokkaido/13/00 (H9N2) for A/duck/Hokkaido/Vac-2/04 (H7N7)) and incubated at room temperature for one hour prior to plaque cloning of the viruses in MDCK cells to neutralize the viruses which have undesired hemagglutinin (HA) and/or neuraminidase (NA) (e.g., H7HA and N2NA in generation of A/duck/Hokkaido/Vac-1/04 (H5N1)). Cloned viruses were subtyped using hemagglutinin-inhibition (HI)[11] and neuraminidase-inhibition (NI) tests[2].

Plaque assay: Purification of viruses by plaque selection method was performed as described previously[5]. Briefly, confluent MDCK cells were incubated one hour at 35℃ with ten-fold dilutions of virus. The cells were then washed and overlaid with MEM (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 0.7% Bacto-agar (Difco, Sparks, MD, U.S.A) and 0.5μg/ml of trypsin and incubated at 35℃. After three days, plaques were picked to be further purified.

Sequencing: Viral RNAs were extracted from infectious allantoic fluids using a commercial kit (TRI LS reagent, Sigma-Aldrich, St. Louis, MO, U.S.A) and reverse-transcribed with the Uni12 primer[6] and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A). Viral complementary DNA was amplified with the polymerase chain reaction (PCR) with the RNA LA PCR Kit Ver. 1.1 (Takara, Shiga, Japan) and PTC-200 thermal cycler (Biorad, Alfred Novel Drive Hercules, CA, U.S.A). The following primers were used or designed on the basis of published nucleotides sequences of HA genes for RT-PCR in the present study; (BmHA

-1F[6]) 5'-TATTCGTCTCAGGGAGCAAAAGCAGGG
G-3', (H5-1695R) 5'-CGATCCATTGGAGCACATCC
-3',and (H7-1141R) 5'-CGATCCATTGGAGCACAT
CC-3'. For sequencing internal protein genes, gene
-specific primer sets were used as described previ-
ously[9]. Nucleotide sequences were determined
from these RT-PCR products using CEQ2000 auto-
mated DNA sequencer (Beckman Coulter, Fuller-
ton, CA, U.S.A) according to Dye Terminater Cycle
Sequence Chemistry Protocol (Beckman Coulter).
Sequence data were analysed using GENETYX
Version 7.0 (Genetyx, Tokyo, Japan).

Pathogenicity studies: Mean death times (MDTs)
of chicken embryos were determined as described
previously[14]. Nine-day-old embryonated chicken
eggs were inoculated with ten-fold dilutions of the
viruses. Eggs were examined every eight hours by
candling; the integrity of the circulatory system
and movement of the embryo were the criteria for
viability. Any embryos which were dead at 24 hour
post-inoculation were discarded as nonspecific
deaths and did not figure in the calculation of
MDT. The MDT was calculated as the mean time
in hours for the minimum dose lethal to kill the
embryos. The intracerebral pathogenicity index
(ICPI) and the intravenous pathogenicity index
(IVPI) tests were performed as described else-
where[1,4]: each infectious allantoic fluid was inocu-
lated into groups of ten one-day-old chicks and
eight six-week-old chickens, via the intracerebral
and intravenous route, respectively. In both stud-
ies, the birds were observed for clinical symptoms
and mortality at 24-hour intervals for a period of
eight days for the ICPI test and ten days for the
IVPI test. The ICPI and IVPI values were calcu-
lated as described[1].

Viral Growth Kinetics: Viruses of 10^2 and 10^4 50%
egg infectious dose (EID_{50}) ($10^{1.5}$ and $10^{3.5}$ EID_{50} for
A/duck/Hokkaido/Vac-2/04 (H7N7)) were inocu-
lated into ten-day-old embryonated chicken eggs
and incubated at 34℃ (35℃ for A/duck/Hokkaido/
Vac-3/07 (H5N1)). Allantoic fluids were harvested
to determine viral titers at different time points (0,

12, 24, 48, and 72 hour). The EID_{50} and hemagglu-
tination titers of each virus present in the allantoic
fluid was determined.

Results

*Generation of non-pathogenic H5N1 strains of Eur-
asian lineage:*

Until now, non-pathogenic H5N1 viruses of
Eurasian lineage have been rarely isolated from
natural hosts[12]. To establish H5N1 vacccine candi-
date strains against H5N1 influenza viruses circu-
lating worldwide, A/duck/Hokkaido/Vac-1/04 (H5N
1) [Vac-1/04 (H5N1)] and A/duck/Hokkaido/Vac-3/
07 (H5N1) [Vac-3/07 (H5N1)] were generated by
the standard genetic reassortant procedure from
non-pathogenic viruses of Eurasian lineage de-
rived from waterbirds and origins of the internal
proteins of these reassortant viruses were deter-
mined (Fig. 1A and 1B). The PB2, PB1, PA, HA,
NP, and M gene segments of Vac-1 (H5N1) were de-
rived from A/duck/Mongolia/54/01 (H5N2), and the
NA and NS gene segments, from A/duck/Mongolia/
47/01 (H7N1) (Fig. 1A). The HA and NS gene seg-
ments of Vac-3 (H5N1) were derived from A/duck/
Hokkaido/101/04 (H5N3), while the other seg-
ments were from A/duck/Hokkaido/262/04 (H6N1)
(Fig. 1B). The complete nucleotide sequences of
Vac-1 (H5N1) and Vac-3 (H5N1) obtained in the
present study have been registered in GenBank
(Accession numbers: AB259709-AB259716, and
AB355926-AB355933).

*Generation of a non-pathogenic H7N7 virus with
good growth potential:*

Dozens of non-pathogenic H7N7 viruses have
been isolated from fecal samples of waterbirds dur-
ing our surveillance work since 2001 (data not
shown). Based on the results of phylogenetic and
antigenic analyses (data not shown), A/duck/Mon-
golia/736/02 (H7N7) [Mon/736 (H7N7)] was se-
lected as a vaccine candidate. However, the
growth potential of Mon/736 (H7N7) in chicken
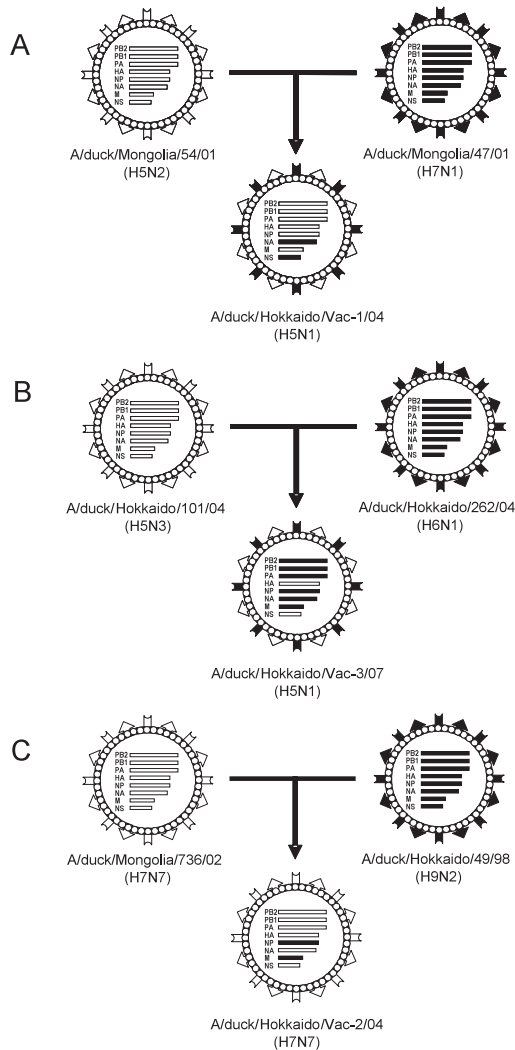embryonated eggs was poor in terms of the HA

Fig. 1. Establishment of candidate strains for vaccines against H5 and H7 highly pathogenic avian influenza viruses by genetic reassortment. Vac-1/04 (H5N1), Vac-3/07 (H5N1), and Vac-2/04 (H7N7) were generated from two non-pathogenic avian influenza viruses isolated from migratory ducks. The origin of viral segments of reassortant viruses is indicated with white and black bands. (A); Vac-1/04 (H5N1) was a reassortant generated with A/duck/Mongolia/54/01 (H5N2) as the HA gene provider and A/duck/Mongolia/47/01 (H7N1) as the NA gene provider. (B); Vac-3/07 (H5N1) was generated with A/duck/Hokkaido/101/04 (H5N3) as the HA gene provider and A/duck/Hokkaido/262/04 (H6N1) as the NA gene provider. (C); Vac-2/04 (H7N7) was generated from Mon/736 (H7N7) as the HA and NA gene provider and Hok/49 (H9N2) to give high growth potential in embryonated eggs.

titer and yield of viral protein. To improve the growth potential of Mon/736 (H7N7), A/duck/Hokkaido/Vac-2/04 (H7N7) [Vac-2/04 (H7N7)] was generated as a genetic reassortant virus between Mon/736 (H7N7) and A/duck/Hokkaido/49/98 (H9N2) [Hok/49 (H9N2)], which possesses good growth potential in embryonated eggs (Fig. 1C). The PB2, PB1, PA, HA, NA, and NS gene segments of Vac-2/04 (H7N7) were derived from Mon/736 (H7N7), and the other segments, from Hok/49 (H9N2) (Fig. 1C).

The complete nucleotide sequences of Vac-2 (H7N7) obtained in the present study have been registered in GenBank (Accession numbers: AB243417-AB243424).

Pathogenicity of vaccine candidate strains:

The pathogenicity of the vaccine candidate strains for chickens, chicks, and chicken embryos was evaluated by measuring the IVPI, ICPI, and MDT. All of the chickens inoculated intravenously with Vac-1 (H5N1), Vac-3 (H5N1), or Vac-2 (H7N7) survived for ten days without showing any clinical signs, and IVPI values were 0.00 (Table 1). The pathogenicity of Vac-1 (H5N1) and Vac-2 (H7N7) for chicks was very low (the ICPI values were 0.10 and 0.20, respectively). Although HAs of all the vaccine candidate strains had typical sequences found in the HA of avirulent type at the cleavage sites as shown in Table 1, half of the chicks inoculated intracerebrally with Vac-3 (H5N1) were dead at two to eight days post-inoculation (ICPI, 0.58), indicating a relatively high pathogenicity for chicks as compared with the other two candidate strains. The MDT of Vac-1 (H5N1), Vac-3 (H5N1), and Vac-2 (H7N7) was 64, 73, and 81 hours, respectively.

Growth character of vaccine candidate strains in chicken embryos:

The growth kinetics of vaccine candidates was determined by using chicken embryos. As shown in Table 2, all the candidates replicated well, and reached plateau by 48 hours. There was no significant difference in peak titers regardless of the in-

Table 1. Pathogenicity of vaccine candidate strains for chickens and chicken embryos

Viruses	Amino acid sequence of HA cleavage site	IVPI[a]	ICPI[b]	MDT[c] (hr)
Vac-1/04 (H5N1)	PQRETR/G	0.00	0.10	64
Vac-3/07 (H5N1)	PQRETR/G	0.00	0.58	73
Vac-2/04 (H7N7)	PEIPKGR/G	0.00	0.20	81

[a] Intravenous pathogenicity index
[b] Intracerebral pathogenicity index
[c] Mean death time

Table 2. Growth kinetics of vaccine candidate strains in chicken embryos[a]

Incubation time (hr)	Vac-1/04 (H5N1)				Vac-3/07 (H5N1)				Vac-2/04 (H7N7)			
	$10^{2.0\,b}$		$10^{4.0}$		$10^{2.0}$		$10^{4.0}$		$10^{1.5}$		$10^{3.5}$	
	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer	Virus titer	HA titer
0	≤ 0.5	<2	≤ 0.5	<2	≤ 0.5	<2	≤ 0.5	<2	≤ 0.5	<2	≤ 0.5	<2
12	3.7	<2	4.5	<2	2.3	<2	5.5	<2	3.5	<2	5.1	<2
24	8.7	32	9.5	256	8.3	512	8.4	1024	7.9	4	8.7	64
48	9.3	256	9.5	512	9.3	2048	9.5	2048	9.5	512	9.1	512
72	9.1	512	9.3	256	9.5	2048	9.5	2048	9.1	512	9.3	512

[a] The virus titers ($logEID_{50}$/ml) and HA titers (HAU/ml) were deterimined from allantoic fluids of chicken embryos inoculated with Vac-1/04 (H5N1), Vac-3/07 (H5N1), and Vac-2/04 (H7N7), respectively.
[b] Dose of inoculum given to chicken embryo (EID_{50}/egg)

oculum dose of each vaccine candidate.

Discussion

It is required for influenza vaccine strain to be antigenically similar to endemic virus strains, non-pathogenic for chicken embryos, and high yield in the embryonated eggs. In response to the 2004 outbreaks of HPAI in Japan, the commercial vaccines were emergently imported from Mexico. However, these vaccines were produced using H5N2 viruses of North American lineage, which are antigenically different from current HPAI viruses circulating in countries in Asia, Central East, Europe, and Africa. It is, therefore, required for the stockpile vaccine that is prepared from virus strains of Eurasian lineage, causing the present epidemics. In the present study, we selected the virus strains isolated from feral waterbirds as H5 or H7 HA providers based on the following criteria: (a) antigenically and genetically similar to HPAI virus presently circulating, (b) the latest isolates, then applied for generation of vaccine candidate strains.

To obtain non-pathogenic H5N1 strains for vaccines, Vac-1 (H5N1) and Vac-3 (H5N1) were generated by genetic reassortment. These two strains showed low pathogenicity in chickens and chicken embryos (Table 1). Additionally, they grew well in embryonated eggs after 48 hours post-inoculation, relatively shorter than their MDT (Table 2), indicating that these virus strains are useful for vaccine production against H5N1 influenza virus infections. Since Vac-3 (H5N1) showed comparatively pathogenic to chicks compared with Vac-1(H5N1) (Table 1), we set Vac-1 (H5N1) as the first candidate strain for H5N1 influenza virus vaccine. The vaccine for chickens by using Vac-1 (H5N1) has been produced in collaboration with vaccine producers and its protective effect is under investigation.

Vac-2 (H7N7) was obtained as a vaccine candidate strain against H7N7 viruses (Fig. 1C). Mon/736 (H7N7), providing the HA and NA gene of Vac-2 (H7N7), showed poor growth potential in chicken

embryos (16HA). To solve this problem, a genetic reassortant was generated using Hok/49 (H9N2), which has high growth potential in eggs. The NP and M gene segments of Vac-2 (H7N7) were derived from Hok/49 (H9N2), indicating that the NP and/or M gene segments were responsible for the growth potential. To determine whether NP and/or M proteins are truly involved in growth potential in chicken embryos or not, further investigation is underway.

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