

# H5 and H9 subtypes of Avian Influenza Viruses are Real Threat To Humans

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Recent outbreaks of H5N1 avian influenza viruses in most Asian countries alert the imminent pandemic. Twenty-three humans out of 34 confirmed cases were dead of H5N1 infections. H9N2 avian influenza viruses are widespread in poultry in most Asian countries and infected humans in 1999 and 2003. H9N2 or H5N1 influenza viruses may cause a next pandemic. The extensive surveillance in poultry and pigs are very important for predicting a next pandemic. We performed the surveillance of avian influenza viruses in chickens sold in the live poultry markets. Only H9N2 subtypes of influenza viruses were isolated from chickens in the Korean live bird markets. Serological surveillance in chickens showed that chickens were infected with over 50% with H9N2 viruses. Antigenic analysis showed that current circulating H9N2 influenza viruses are distinct from those of Hong Kong and 1996 Korean isolates from chicken farms. Serological surveillance of pigs against H9N2 influenza viruses showed that over 20% are positive. To prepare the vaccine of H5N1 avian influenza viruses, reassortant viruses were made using the available reverse genetics. The efficacy and safety test of H5N1 candidate vaccine in monkeys showed that neutralization antibody were induced and no side effects such as fever and weight loss were observed.

## Introduction

Influenza virus pandemics can occur in the interval of 10-30 years and will cause devastating effects on humans (1,2). They usually arise as a result of introducing the genes of avian influenza viruses into the human influenza viruses. In the 20th century, humans had experienced three major pandemics, starting in 1918 caused by an H1N1 subtype, in 1957 by an H2N1 subtype, and in 1968 by an H3N2 subtype. The 1968 H3N2 pandemic virus had emerged by introducing avian PB1 and hemagglutinin (HA) gene into the H2N2 human influenza viruses in pigs. This indicates that the continuous surveillance of avian influenza viruses in poultry and pigs is very important for predicting a new pandemic.

In 1997, the highly pathogenic H5N1 influenza viruses were directly transmitted from chickens in the Hong Kong live bird markets to humans and claimed six human lives out of 18 infected cases (3-5). In 1999, H9N2 influenza viruses infected two children and in 2003, H9N2 influenza

viruses infected one child in Hong Kong (5-7). Considering that poultry in the live bird markets closely contact humans every day, the surveillance of live bird markets can be very important for monitoring the new subtypes of influenza viruses in humans.

At the event of pandemic, each country may face the serious vaccine shortage since the current egg production systems may not produce vaccine enough to cover all humans. In addition, many countries including Korea do not have infrastructure to produce a pandemic vaccine. The candidate vaccine preparedness for humans is urgent and each nation should decide to set up the emergency infrastructure since a pandemic is imminent.

## Methods

### 1. Surveillance of chickens in the live poultry markets

We visited the live poultry markets in Korea and picked up feces samples of the poultry in PBS with antibiotics.

The samples were directly shipped into the laboratory and were inoculated into 10-day-old embryonated eggs for the isolation of avian influenza viruses.

## 2. Hemagglutination inhibition (HI) assay for serological surveillance.

In 96-well plates, 50  $\mu$ l of sera (1:10) were added to a well and then 2-fold dilutions were performed in 25  $\mu$ l of PBS. 25  $\mu$ l of influenza viruses was added to each well in plates and plates were incubated at room temperature for 15 min before 50  $\mu$ l of 0.5% chicken RBC in PBS were added to wells. HI titers were recorded at 40 minutes after RBC was added.

## 3. Generation of recombinant viruses

For the generation of recombinant vaccine viruses, the eight reverse genetics plasmids of H1N1 human influenza viruses were used. HA of the highly pathogenic H5N1 viruses were amplified by PCR and the polybasic amino acids were removed by PCR before HA was cloned into pHW2000 vector. Cloned HA plasmid with seven H1N1 human plasmids were transfected into 293-T cells to create the recombinant H5N1 vaccine candidate.

Neutralization Assay. Vaccine efficacy was measured by viral neutralization assay in MDCK cells. Sera were two-fold diluted before mixed with H5N1 viruses (100 TCID<sub>50</sub>). Mixed viruses were added into MDCK cells in 96-well plates for 48 hours before the viral presence and neutralization titers were determined.

## Results

### 1. Subtypes of avian influenza viruses from chickens in Korean live bird markets

There has been limited information on avian influenza viruses from chickens in Korean live bird markets. To understand what subtypes of viruses are circulating among chickens in Korean live bird markets, we surveyed chickens in the live bird markets. We could isolate 8 H9N2 influenza viruses from 95 chicken samples (Table 1).

### 2. Serological surveillance in chickens

We are interested in determining the serological prevalence in chickens in Korea. One hundred sera were collected from chickens from live bird markets in Korea before HI assays were performed. Over 50% of chicken sera were positive for H9N2 influenza viruses. No reactivity was detected for an H6N1 or H3N2 influenza virus. HI titers over 40 were regarded as positive samples (Table 2).

### 3. Antigenic comparison

To study the serological similarity of H9N2 influenza viruses isolated from the Korean live bird markets to other H9N2 isolates from other countries, we made immune sera against A/Chicken/Korea/S1/03 (H9N2) by inoculating chickens before HI assay was carried out. HI titers were 640 against A/Chicken/Korea/S1/03, 80 against A/Turkey/WI/66, 10 against A/Hong Kong /1073/99, 160 against A/Chicken/Korea/96 (H9N2), and 20 against A/Duck/Hong Kong/Y280/97. The results indicate that current H9N2 influenza viruses isolated from chickens in Korea are serologically different from those of Hong Kong isolates and 1996 Korean isolates (Table 3).

### 4. Serological surveillance of influenza viruses in pigs

Pigs are postulated to be "mixing vessels" for creating pandemic influenza viruses since pigs have both avian and human receptors for influenza infections. Pigs are currently

**Table 1. Subtypes of avian influenza viruses isolated from chicken in Korean live bird markets**

Subtypes	Poultry (#Positive/total isolates)	
	Chickens	
H9N2	8/8	
H6N1	-	
H3N2	-	

**Table 2. Serological surveillance in chickens**

Subtypes	Numbers of Chickens
	(#Positive/total samples)
H9N2	52/100
H6N1	0/100
H3N2	0/100

**Table 3. Serological similarity among H9N2 influenza virus**

Viruses				
A/Chicken/Korea/S1/03	A/Turkey/W1/66	A/Hong Kong/1073/99	A/Duck/Hong Kong/Y280/97	A/Chicken/Korea/96
640	80	10	20	160

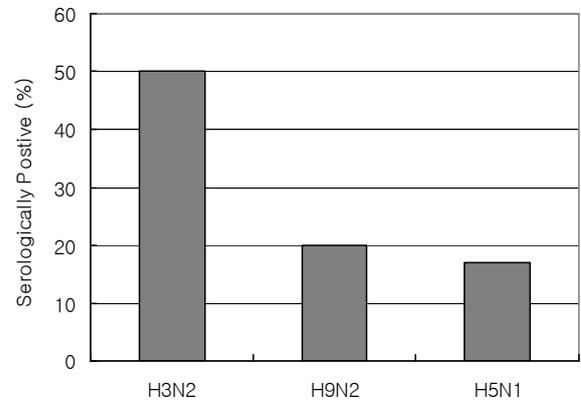
infected with H1N1 and H3N2 influenza viruses as in humans. To determine whether pigs are infected with H9N2 or H5 subtypes of influenza viruses, we performed the serological surveillance. About 200 sera were tested. The positive percent against antigens, H3N2, H9N2 and H5N1 was about 50, 20, and 17, respectively (Fig. 1).

**5. Vaccine efficacy test of a candidate H5N1 influenza virus in monkeys**

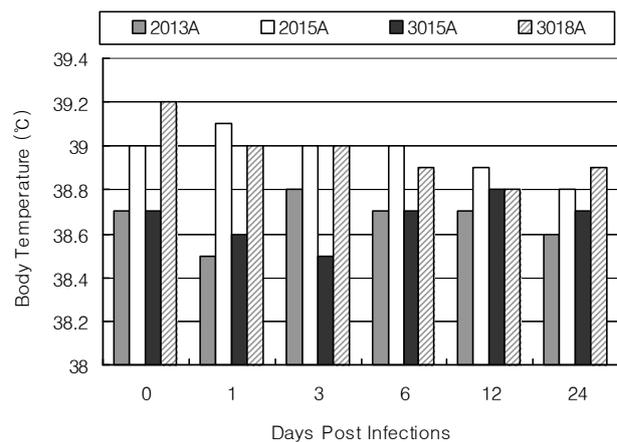
H5N1 subtypes of influenza viruses may be a next pandemic strain. The candidate H5N1 vaccine strain was tested in monkeys for efficacy and safety. Four African green monkeys were infected i.m. with 15 µg of HA of H5N1 vaccine strain. The range of body temperature before inoculation was 38.7 to 39.2 and that of vaccinated monkeys was from 38.5-39.1 (Fig. 2). To determine the vaccine efficacy, the neutralization titers against H5N1 influenza viruses were measured. The titers of neutralization antibody at 14 days p.i. were less than 40. The second vaccine was inoculated at 14 days after the first inoculation. Sera were collected at 6 days after the second inoculation and neutralization antibodies were measured. The range of titers was 80-320 (Fig. 3). Our data suggest that H5N1 candidate vaccine may be efficacious in humans.

**Discussion**

Live bird markets seem to play an important role in transmission of avian influenza viruses to humans since live bird markets are ideal places where humans can closely contact live birds. In 1997, the highly pathogenic H5N1 viruses were transmitted to humans, killing six human lives (3). In 1999 and 2003, avian H9N2 influenza viruses were transmitted from chickens to humans. These incidents indicate that avian influenza viruses can infect humans and cause the disease (5). In Korea, there are several hundred live bird markets scattered throughout eight



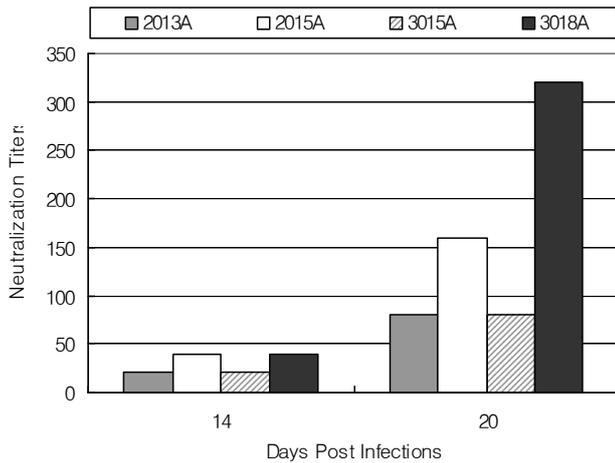
**Fig. 1.** Serological reactivity of porcine sera. About 200 porcine sera were collected from the local farms and the serological reactivity was determined against antigens, H3N2, H9N2, or H5N1 by HI assay. Over 40 was regarded as positive.



**Fig. 2.** Body temperature in monkeys. Four African green monkeys were inoculated with H5N1 vaccine and body temperatures measured before inoculation and after inoculation.

provinces. There are two types of live bird markets, five-day interval and sentinel markets in Korea. The live birds are sold when markets open every 5 days in five-day markets and sentinel markets sell live birds every day.

Antigenic study suggests that H9N2 Korean isolates are different from those of Hong Kong. In Hong Kong live bird markets, in 1997, G1 and Y280 lineage H9N2 viruses circulated in the Hong Kong live bird markets. Results



**Fig. 3.** Efficacy of H5N1 vaccine in monkeys. Monkeys were inoculated two times at the intervals of 14 days. Sera were collected at 14 days and 20 days p.i. Neutralization assay were performed in MDCK cells with H5N1 viruses.

indicate that the origins of Korean isolates are different from those of Hong Kong H9N2 isolates. The antigenic comparison with 1996 Korean isolates suggests that current circulating H9N2 influenza viruses undergo the antigenic drift.

The serological reactivity of porcine sera against antigens, H5N1 and H9N2 is of great concern. The last two pandemics were created in pigs where the co-infections of human and avian influenza viruses occurred in 1957 and 1968. Human influenza-like H3N2 are circulating in pigs. Our results warrant the continual surveillance of pigs to predict the next pandemic strains.

Our candidate vaccines were efficacious in monkeys. Considering that monkeys are very close to humans, our data are very promising. When H5N1 viruses gain the human to human transmission, thousands of humans will be dead. Our candidate vaccine may be used for human protections.

In conclusion, a pandemic is inevitable and unpredictable. The consequences of pandemic must never be underestimated. We must prepare for all the possible things to protect humans from the imminent pandemic.

## Acknowledgment

This work was supported by Ellison Medical Foundation, USA. We greatly appreciate Dr. Robert G. Webster for providing us with standard sera for avian influenza viruses.

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