Antibody survey on avian influenza viruses using egg yolks of ducks in Hanoi between 2010 and 2012

Kozue Hotta\textsuperscript{a,b,*}, Hiroki Takakuwa\textsuperscript{c}, Toshiyo Yabuta\textsuperscript{c}, Trang T.H. Ung\textsuperscript{b}, Tatsufumi Usui\textsuperscript{c}, Hang L.K. Nguyen\textsuperscript{d}, Thanh T. Le\textsuperscript{d}, Mai Q. Le\textsuperscript{d}, Tsuyoshi Yamaguchi\textsuperscript{c}, Koichi Otsuki\textsuperscript{c}, Toshihiro Ito\textsuperscript{e}, Toshiyuki Murase\textsuperscript{e} and Tetsu Yamashiro\textsuperscript{a,b}

\textsuperscript{a}Center for Infectious Disease Research in Asia and Africa, Institute of Tropical Medicine (NEKKEN), Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

\textsuperscript{b}Vietnam Research Station, Nagasaki University, c/o National Institute of Hygiene and Epidemiology, No. 1 Yersin street, Hanoi, Vietnam

\textsuperscript{c}Avian Influenza Research centre, Kyoto Sangyo University, Kamigamo-motoyama, Kita, Kyoto 603-8555, Japan

\textsuperscript{d}Department of Virology, National Institute of Hygiene and Epidemiology, No. 1 Yersin street, Hanoi, Vietnam

\textsuperscript{e}Faculty of Agriculture, Tottori University, 4-101 Koyama, Tottori 680-8553, Japan
Abstract

In Vietnam, numerous surveillance programs are conducted to monitor the prevalence of avian influenza (AI) viruses. Three serological methods—the agar-gel immunodiffusion test, hemagglutination inhibition (HI) test, and enzyme-linked immunosorbent assay—are well established for detection of AI virus antibodies in poultry sera. Several recent reports have validated egg yolk as an alternative source for detection of AI virus antibodies. In this study, we investigated AI virus antibodies in ducks by HI testing using egg yolk. Ten duck eggs were collected every month from 10 randomly selected markets in Hanoi from April 2010 to March 2012. The HI test was performed
using low pathogenic avian influenza (LPAI) viruses (H3, H4, H6, H7, H9, and H11 subtypes) and highly pathogenic avian influenza (HPAI) viruses (H5N1 clade 2.3.4 and 2.3.2.1) as antigens. HI testing for H3, H6, and H9 was 29% positive in November 2010, 50% positive in October and November 2010, and 12% positive in June 2011. These results indicated that several epidemics of LPAI viruses had occurred during the study period. In addition, antibodies against H7 were negative. The results of HI testing for H5N1 showed that the reactivity of the dominant HI antibody shifted from H5N1 clade 2.3.4 to clade 2.3.2.1. In conclusion, egg yolk is useful for long term monitoring of AI virus antibodies and the use of egg-based antibody detection may contribute to improvements in animal welfare.

**Keywords**

Avian influenza virus, Egg yolk, Hemagglutination inhibition test

1. **Introduction**
Outbreaks of highly pathogenic avian influenza (HPAI) of the H5N1 subtype have occurred in Vietnam since December 2003 (Hien et al., 2009).

Ducks are of particular concern because they are asymptomatic carriers of avian influenza (AI) viruses including low pathogenic avian influenza (LPAI) and HPAI (Chen et al., 2004; Sturm-Ramirez et al., 2005). Therefore, ducks play an important role in transmission of AI viruses. Recently, various AI viruses were isolated from ducks in Vietnam, including the H3N2, H3N8, H4N6, H5N1, H5N2, H6N1, H9N2, H9N3, H9N6, H11N3, and H11N9 subtypes (Hotta et al., 2012; Nguyen et al., 2005; Nomura et al., 2012).

In 1997, an H5N1 influenza virus outbreak occurred among chickens in Hong Kong, and the virus was transmitted directly to humans. Phylogenetic analysis indicated the highest homology between the internal genes of A/quail/Hong Kong/G1/97 (H9N2), A/teal/Hong Kong/W312/97 (H6N1), and the H5N1 isolates (Guan et al., 1999; Hoffmann et al., 2000). These reports suggest that reassortment occurred between the H9N2, H6N1, and H5N1 viruses, possibility involving the internal genes of the H5N1 virus, which
were acquired from H9N2 and H6N1. To control HPAI viruses and monitor
the generation of novel viruses, surveillance for AI viruses among poultry is
needed in countries where H5N1 strains are circulating.

Sero-epidemiological studies targeting a specific antibody against AI
viruses are commonly used to collect evidence of infection or to evaluate the
effects of vaccination. Because animal welfare is an issue of great concern,
there is a requirement for alternative sources of antibodies that can be
produced without pain and distress to the animals (Silim and Venne, 1989).

In terms of animal welfare as well as economic considerations, the use of egg
yolk antibodies instead of serum is sufficient to allow AI surveillance among
chickens and ducks (Beck et al., 2003; Jeong et al., 2010; Trampel et al.,
2006).

In this study, we examined an egg yolk antibody as an alternative source
for AI virus antibody detection in layer ducks, and antibodies against
hemagglutinin (HA) were used as markers for both infection and vaccination.

Because the vaccine used in northern Vietnam is generated from a
genetically modified reassortant H5N1 virus, differentiation between the virus in infected and vaccinated poultry is difficult when measuring the antibody response against HA. To monitor the prevalence of AI viruses in ducks, we collected duck eggs from markets in Hanoi, and examined hemagglutination inhibition (HI) antibodies using LPAI viruses as antigens. In addition, to investigate whether the reactivity of HI antibodies was different between different clades of H5N1 viruses, we performed HI testing using HPAI H5N1 clade 2.3.4 and 2.3.2.1 viruses as antigens.

2. Materials and methods

2.1. Sample collection and preparation of egg yolk

In total, 2,378 duck eggs were collected in Hanoi from April 2010 to March 2012. Ten eggs were obtained from each of the 10 randomly selected markets every month to yield 100 eggs. For yolk immunoglobulin extraction using a simplified chloroform polyethylene-glycol procedure, 2 ml of egg yolk was mixed with an equal volume of phosphate-buffered saline, and then
added to 4 ml of chloroform (Polson, 1993). After mixing well, the yolk was
centrifuged at 3,500 rpm for 10 min. The supernatant was collected and used
for antibody tests.

2.2. Virus and antigen preparation

LPAI A/duck/Ukraine/1/63 (H3N8), A/duck/Czechoslovakia/1/56 (H4N6),
A/turkey/Massachusetts/3740/65 (H6N2), A/whistling swan/Shimane/35/80
(H6N6), A/whistling swan/Shimane/42/80 (H7N7), A/swan/Shimane/42/99
(H7N8), A/turkey/Massachusetts/3740/65 (H9N2), A/whistling
swan/Shimane/48/97 (H11N2), A/duck/England/1/56 (H11N6), HPAI
A/Vietnam/31244/2007 (H5N1, clade 2.3.4), A/muscovy
duck/Vietnam/LBM57/2011 (H5N1, clade 2.3.2.1), and swine influenza
A/swine/Iowa/15/30 (H1N1) were propagated in 9- to 10-day-old
embryonated chicken eggs. Before using the embryonated eggs, HA testing
was performed to confirm that the eggs did not contain antibodies against
influenza viruses. Viruses in the harvested allantoic fluids were inactivated
with 0.1% formalin (v/v) for 7 days at 4°C. Virus inactivation was confirmed
by 2 blind passages in embryonated eggs.

2.3. HI test

The HI test was performed according to the standard procedures recommended by the World Health Organization. Briefly, the yolk samples were treated with a receptor-destroying enzyme (RDE) (Denka Seiken Co. Ltd., Tokyo, Japan) at 37°C for 20 h to eliminate non-specific inhibitors of hemagglutination. HI titers obtained from a purified yolk and RDE mixture (25 µl egg yolk + 75 µl RDE provided a starting dilution of 1:1) were defined as the reciprocal of the highest dilution of yolk, which completely inhibited hemagglutination of 4 hemagglutination units of the virus with a 0.5% solution of chicken red blood cells. Samples with HI titers under 16 were considered negative.

3. Results and discussion

To determine the prevalence of AI viruses among ducks in Hanoi, HI
testing was performed using 2,378 egg yolks obtained from April 2010 to March 2012. As shown in Table 1, selected samples from the 2,378 egg yolks showed patterns of positive results in the HI test. To confirm the effects of the NA subtype on HI testing, the HI test was performed using H1N1 for H5N1, H7N8 for H3N8, and H11N2 for H6N2 and H9N2. The positive samples did not overlap between H4N6 and H6N6. These results indicated that there was no effect of the NA subtype on HI testing in this study.

From April 2010 to March 2012, the positivity rates of the yolk antibody against LPAI viruses were 1.98% and 7.7% for H3 and H6, respectively. The other subtypes showed lower than 1% positivity (0.25%, 0.42%, and 0.67% for H4, H9, and H11, respectively) (Table 2). An antibody against H7 was not detected in egg yolk. H6 (7.7%) was the most frequently detected HA subtype in ducks.

As shown in Figure 1, several epidemics of LPAI viruses were observed during the monitoring period. The HI antibody against H3 was 29% positive in November 2010, whereas the HI antibody H11 was 12% positive in June.
2011. The HI antibody against the H6 subtype was detected from April 2010 to January 2011, except in August 2010. There was a drastic peak in October and November 2010 during which the yolk-antibody positivity rate was 50%.

Thus far, no vaccination against LPAI viruses has been conducted for domestic poultry in Vietnam. These results indicated that several epidemics of LPAI viruses had occurred among ducks from April 2010 to March 2012.

To investigate whether the reactivity of the HI test was different between different clades of H5N1 subtypes, the HI test was performed using H5N1 clade 2.3.4 and 2.3.2.1 viruses. Positivity rates for yolk antibodies were 23% (547/2,378) for H5N1 clade 2.3.4 and 22% (512/2,378) for H5N1 clade 2.3.2.1 from April 2010 to March 2012 (Table 3). The HI antibody against the H5N1 clade 2.3.4 subtype ranged from 63 to 100% positive during April 2010 to October 2011, but decreased substantially to 33% in November 2011 (Fig. 2).

In contrast, the HI antibody against the H5N1 clade 2.3.2.1 subtype ranged 62 to 97% positive between November 2011 and March 2012. In particular, the HI antibody that reacted with only the H5N1 clade 2.3.2.1 subtype was
increased to 67%, 66%, 77%, 51%, and 59% positive in each consecutive month between November 2011 and March 2012. Statistical analysis using the Fisher’s exact test with the level of significance set at $P < 0.01$ indicated that the positivity rate of the HI antibody for the H5N1 clade 2.3.2.1 subtype was significantly higher in November 2011 and January 2012 than in April 2010. These results indicated that the major HI antibody shifted from positive for H5N1 clade 2.3.4 to clade 2.3.2.1.

Because ducks can be infected with HPAI virus without clinical signs and the virus can be detected only for a short time during shedding, active surveillance is not beneficial for ducks (Spackman et al., 2009). In cases of asymptomatic infection, serological tests are particularly useful to evaluate antibodies for monitoring the prevalence of AI viruses. However, there are practical difficulties in collecting serum from layer ducks. Collecting blood samples from layer ducks is stressful to the ducks, which causes economic losses by reducing egg production. Therefore, it was necessary to establish an alternative source of AI virus antibodies other than serum. Thus far,
some studies have attempted to resolve this issue by using egg yolk. Egg yolk antibodies in chickens are a good alternative source for detection of the AI virus antibody (Beck et al., 2003). Furthermore, as an alternative to serum, egg yolk is a feasible and recommended source for monitoring the AI virus antibody in ducks (Jeong et al., 2010). To our knowledge, this is the first report to detect AI virus antibodies using duck egg yolk for long-term monitoring.

H5N1 vaccination has no effect on LPAI viruses. Therefore, the presence of antibodies against LPAI viruses in ducks indicates infection with LPAI viruses. We found that there were apparent epidemics of H3, H6, and H11 subtypes in Hanoi between April 2010 and March 2012 (Fig. 1). On the other hand, we did not detect the H7 subtype. In this study, we used H5N1 clade 2.3.4 and 2.3.2.1 viruses for the HI antigen. These viruses showed low cross-reactivity in the HI test (Nguyen et al., 2012). Our data showed changes in the reactivity of antibodies against H5N1 viruses in egg yolk (Fig. 2). From April 2010 to October 2011, the HI antibody positivity rate for the
H5N1 clade 2.3.4 subtype was higher than that for clade 2.3.2.1. During this period, ducks infected with the H5N1 clade 2.3.4 virus were difficult to differentiate from vaccinated ducks, because the H5N1 clade 2.3.4 virus is used in the recombinant vaccine. However, the HI antibody positivity rate for the H5N1 clade 2.3.2.1 subtype increased from November 2011 onward until March 2012. It is likely that the alterations in reactivity of the HI antibody occurred in response to changes in the circulation of H5N1 virus in northern Vietnam. Since 2011, there has been no report of H5N1 clade 2.3.4 in Vietnam according to GenBank. Taken together, the results of antibody detection in egg yolks indicated a shift from clade 2.3.4 to 2.3.2.1 of the H5N1 virus dominantly circulating in ducks between April 2010 and March 2012.

In conclusion, our results suggest that duck egg yolks are suitable sources for monitoring the prevalence of AI viruses over a long term without the necessity of extracting blood samples from ducks. In particular, the use of eggs addresses both animal welfare and economic concerns. Since March 2013, the H7N9 subtype has been detected in China (Gao et al., 2013), and
Vietnam is a neighboring country. The H7N9 virus is asymptomatic in poultry, and it is difficult to monitor H7N9 virus in poultry. Therefore, the surveillance of H7N9 virus is a very important issue in Vietnam. Egg yolk is a more practical source for the surveillance of AI virus antibodies in poultry. We are continuing to survey AI viruses using egg yolk for H7N9 monitoring.

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References


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Figure 1. Positivity rate of HI antibodies against LPAI viruses in duck egg yolk from April 2010 to March 2012

Figure 2. Positive number of duck egg yolks in HI testing using H5N1 HPAI viruses from April 2010 to March 2012
Columns indicate HI antibody positivity for only H5N1 clade 2.3.4 (black), both H5N1 clades 2.3.4 and 2.3.2.1 (gray), and only H5N1 clade 2.3.2.1 (white).
Fig. 1
Fig. 2

The bar chart illustrates the distribution of positive yolk samples from different clades over the years 2010 and 2011. The x-axis represents the months from April to March, while the y-axis indicates the number of positive yolk samples. The chart is color-coded to distinguish between clade 2.3.2.1, clade 2.3.4, and clade 2.3.4 and 2.3.2.1. The data points are listed in the table below the chart:

- **clade 2.3.2.1**: 4, 6, 0, 4, 0, 0, 8, 18, 12, 5, 5, 3, 4, 3, 11, 6, 20, 30, 20, 36, 19, 20
- **clade 2.3.4 and 2.3.2.1**: 5, 2, 6, 13, 9, 0, 6, 36, 23, 9, 15, 18, 7, 10, 16, 14, 23, 10, 3, 11, 4, 8, 4, 13
- **clade 2.3.4**: 8, 35, 34, 20, 13, 14, 16, 6, 21, 8, 18, 17, 3, 4, 6, 3, 13, 2, 12, 4, 7, 3, 14, 1
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<td>1698</td>
<td>August, 2011</td>
<td>Kim Lien</td>
<td>&lt;</td>
<td>&lt;</td>
<td>32</td>
<td>8</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>&lt;</td>
</tr>
<tr>
<td>2335</td>
<td>March, 2012</td>
<td>Ha Dong</td>
<td>1024</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>&lt;</td>
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<tr>
<td>Control***</td>
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</tr>
</tbody>
</table>

*, H5N1 clade 2.3.2.1; **, H5N1 clade 2.3.4; ***, eggs from duck which was no infection with avian influenza viruses; <, HI titer of <1:8
**Table 2**
Detection rates of specific antibodies in duck egg yolk against LPAI viruses from April 2010 to March 2012.

<table>
<thead>
<tr>
<th>Subtypes used for antigen</th>
<th>H3</th>
<th>H4</th>
<th>H6</th>
<th>H7</th>
<th>H9</th>
<th>H11</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of positive samples</td>
<td>47 (1.98%)</td>
<td>6 (0.25%)</td>
<td>183 (7.7%)</td>
<td>0</td>
<td>10 (0.42%)</td>
<td>16 (0.67%)</td>
</tr>
<tr>
<td>No. of negative samples</td>
<td>2331</td>
<td>2372</td>
<td>2195</td>
<td>2378</td>
<td>2368</td>
<td>2362</td>
</tr>
<tr>
<td>No. of total samples</td>
<td>2378</td>
<td>2378</td>
<td>2378</td>
<td>2378</td>
<td>2378</td>
<td>2378</td>
</tr>
</tbody>
</table>
Table 3

Detection rates of specific antibodies in duck egg yolk against H5N1 viruses from April 2010 to March 2012.

<table>
<thead>
<tr>
<th>H5N1 clade 2.3.4</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>265</td>
<td>282</td>
<td>547</td>
</tr>
<tr>
<td>(11.1%)</td>
<td>(11.9%)</td>
<td>(23%)</td>
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</tr>
<tr>
<td>Negative</td>
<td>247</td>
<td>1584</td>
<td>1831</td>
</tr>
<tr>
<td>(10.4%)</td>
<td>(66.6%)</td>
<td>(77%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>512</td>
<td>1866</td>
<td>2378</td>
</tr>
<tr>
<td>(21.5%)</td>
<td>(78.5%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>