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

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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

65 were acquired from H9N2 and H6N1. To control HPAI viruses and monitor
66 the generation of novel viruses, surveillance for AI viruses among poultry is
67 needed in countries where H5N1 strains are circulating.

68 Sero-epidemiological studies targeting a specific antibody against AI
69 viruses are commonly used to collect evidence of infection or to evaluate the
70 effects of vaccination. Because animal welfare is an issue of great concern,
71 there is a requirement for alternative sources of antibodies that can be
72 produced without pain and distress to the animals (Silim and Venne, 1989).
73 In terms of animal welfare as well as economic considerations, the use of egg
74 yolk antibodies instead of serum is sufficient to allow AI surveillance among
75 chickens and ducks (Beck et al., 2003; Jeong et al., 2010; Trampel et al.,
76 2006).

77 In this study, we examined an egg yolk antibody as an alternative source
78 for AI virus antibody detection in layer ducks, and antibodies against
79 hemagglutinin (HA) were used as markers for both infection and vaccination.
80 Because the vaccine used in northern Vietnam is generated from a

81 genetically modified reassortant H5N1 virus, differentiation between the
82 virus in infected and vaccinated poultry is difficult when measuring the
83 antibody response against HA. To monitor the prevalence of AI viruses in
84 ducks, we collected duck eggs from markets in Hanoi, and examined
85 hemagglutination inhibition (HI) antibodies using LPAI viruses as antigens.
86 In addition, to investigate whether the reactivity of HI antibodies was
87 different between different clades of H5N1 viruses, we performed HI testing
88 using HPAI H5N1 clade 2.3.4 and 2.3.2.1 viruses as antigens.
89
90 **2. Materials and methods**
91 *2.1. Sample collection and preparation of egg yolk*
92 In total, 2,378 duck eggs were collected in Hanoi from April 2010 to
93 March 2012. Ten eggs were obtained from each of the 10 randomly selected
94 markets every month to yield 100 eggs. For yolk immunoglobulin extraction
95 using a simplified chloroform polyethylene-glycol procedure, 2 ml of egg yolk
96 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

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

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

142 As shown in Figure 1, several epidemics of LPAI viruses were observed
143 during the monitoring period. The HI antibody against H3 was 29% positive
144 in November 2010, whereas the HI antibody H11 was 12% positive in June

145 2011. The HI antibody against the H6 subtype was detected from April 2010
146 to January 2011, except in August 2010. There was a drastic peak in October
147 and November 2010 during which the yolk-antibody positivity rate was 50%.
148 Thus far, no vaccination against LPAI viruses has been conducted for
149 domestic poultry in Vietnam. These results indicated that several epidemics
150 of LPAI viruses had occurred among ducks from April 2010 to March 2012.

151 To investigate whether the reactivity of the HI test was different between
152 different clades of H5N1 subtypes, the HI test was performed using H5N1
153 clade 2.3.4 and 2.3.2.1 viruses. Positivity rates for yolk antibodies were 23%
154 (547/2,378) for H5N1 clade 2.3.4 and 22% (512/2,378) for H5N1 clade 2.3.2.1
155 from April 2010 to March 2012 (Table 3). The HI antibody against the H5N1
156 clade 2.3.4 subtype ranged from 63 to 100% positive during April 2010 to
157 October 2011, but decreased substantially to 33% in November 2011 (Fig. 2).
158 In contrast, the HI antibody against the H5N1 clade 2.3.2.1 subtype ranged
159 62 to 97% positive between November 2011 and March 2012. In particular,
160 the HI antibody that reacted with only the H5N1 clade 2.3.2.1 subtype was

161 increased to 67%, 66%, 77%, 51%, and 59% positive in each consecutive
162 month between November 2011 and March 2012. Statistical analysis using
163 the Fisher's exact test with the level of significance set at $P < 0.01$ indicated
164 that the positivity rate of the HI antibody for the H5N1 clade 2.3.2.1 subtype
165 was significantly higher in November 2011 and January 2012 than in April
166 2010. These results indicated that the major HI antibody shifted from
167 positive for H5N1 clade 2.3.4 to clade 2.3.2.1.

168 Because ducks can be infected with HPAI virus without clinical signs and
169 the virus can be detected only for a short time during shedding, active
170 surveillance is not beneficial for ducks (Spackman et al., 2009). In cases of
171 asymptomatic infection, serological tests are particularly useful to evaluate
172 antibodies for monitoring the prevalence of AI viruses. However, there are
173 practical difficulties in collecting serum from layer ducks. Collecting blood
174 samples from layer ducks is stressful to the ducks, which causes economic
175 losses by reducing egg production. Therefore, it was necessary to establish
176 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

193 H5N1 clade 2.3.4 subtype was higher than that for clade 2.3.2.1. During this
194 period, ducks infected with the H5N1 clade 2.3.4 virus were difficult to
195 differentiate from vaccinated ducks, because the H5N1 clade 2.3.4 virus is
196 used in the recombinant vaccine. However, the HI antibody positivity rate
197 for the H5N1 clade 2.3.2.1 subtype increased from November 2011 onward
198 until March 2012. It is likely that the alterations in reactivity of the HI
199 antibody occurred in response to changes in the circulation of H5N1 virus in
200 northern Vietnam. Since 2011, there has been no report of H5N1 clade 2.3.4
201 in Vietnam according to GenBank. Taken together, the results of antibody
202 detection in egg yolks indicated a shift from clade 2.3.4 to 2.3.2.1 of the H5N1
203 virus dominantly circulating in ducks between April 2010 and March 2012.
204 In conclusion, our results suggest that duck egg yolks are suitable sources
205 for monitoring the prevalence of AI viruses over a long term without the
206 necessity of extracting blood samples from ducks. In particular, the use of
207 eggs addresses both animal welfare and economic concerns. Since March
208 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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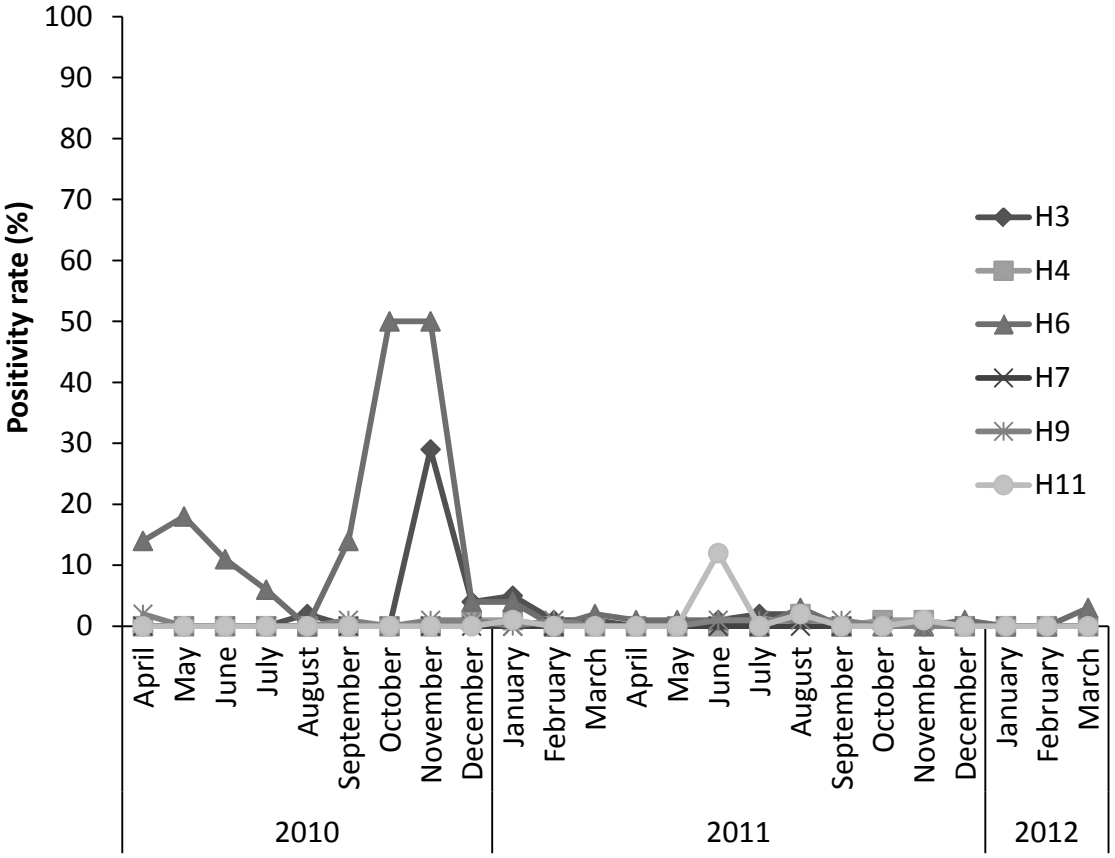
Figure 1. Positivity rate of HI antibodies against LPAI viruses in duck egg yolk from April 2010 to March 2012

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


Positivity rate (%)
100
90
80
70
60
50
40
30
20
10
0
H3
H4
H6
H7
H9
H11
April
May
June
July
August
September
October
November
December
January
February
March
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May
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July
August
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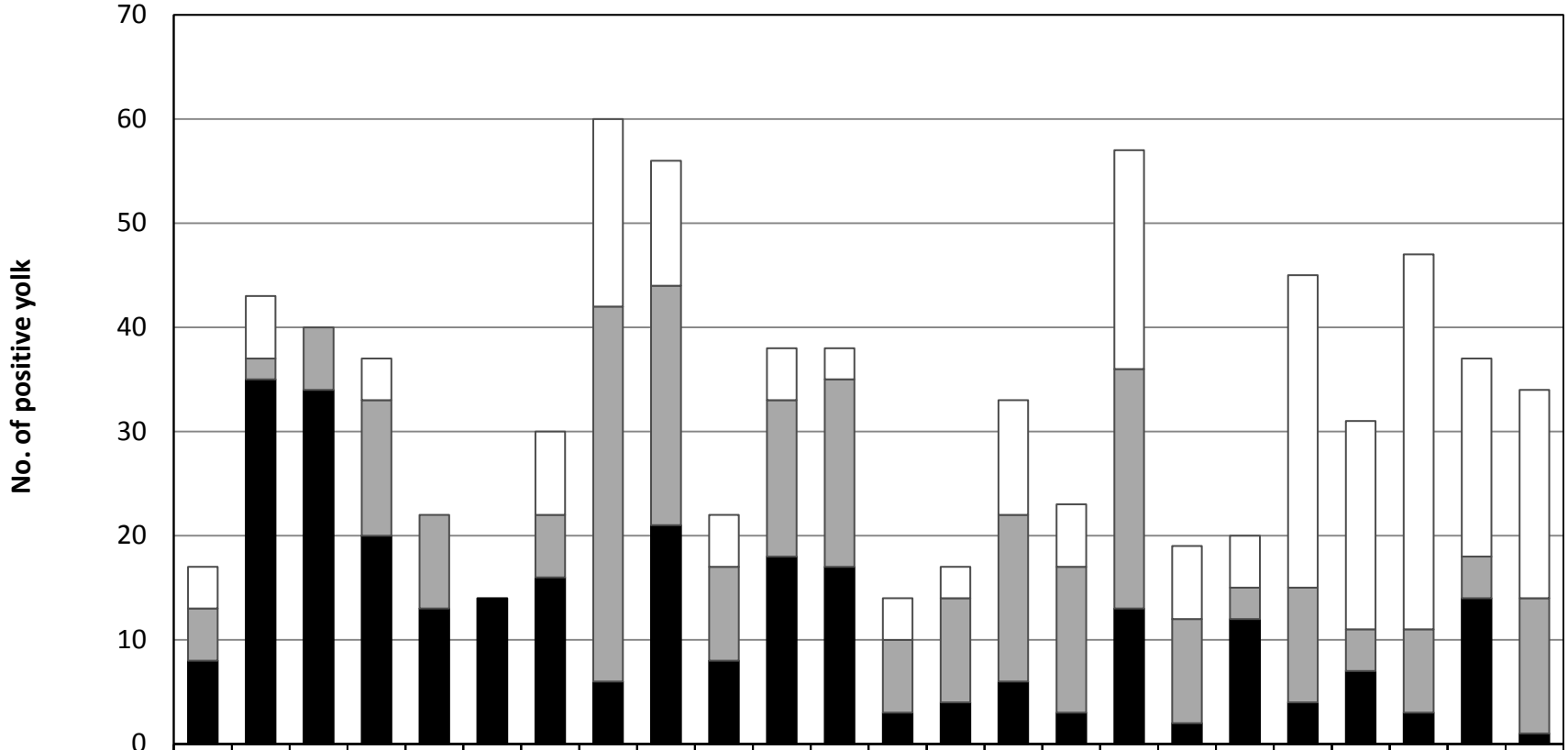
2010
2011
2012

Fig. 2



	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar
	2010									2011												2012		
□ clade 2.3.2.1	4	6	0	4	0	0	8	18	12	5	5	3	4	3	11	6	21	7	5	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

Table 1

HI titers and cross-reactivity of HI test against avian and swine influenza viruses in duck egg yolks

Sample no.	Sampling month	Market	HI antigen for this study								HI antigen for NA control		
			2.3.2.1*	2.3.4**	H3N8	H4N6	H6N2	H6N6	H7N7	H9N2	H11N6	H1N1	H7N8	H11N2
2	April, 2010	Hom	<	16	<	<	1024	512	<	<	<	<	<	<
32	April, 2010	DongXuan	<	<	<	<	2048	2048	<	512	<	<	<	<
122	May, 2010	Nguyen Cao	<	8	<	<	2048	<	<	<	<	<	<	<
123	May, 2010	Nguyen Cao	<	16	<	<	2048	<	<	<	<	8	<	<
218	June, 2010	Nguyen Cong Tru	32	32	<	<	32	<	<	<	<	<	<	<
256	June, 2010	Van Quan	<	512	<	<	<	<	<	<	<	<	<	<
340	June, 2010	Hang Be	64	128	<	<	<	<	<	<	<	<	<	<
599	September, 2010	Mo	<	<	<	<	1024	1024	<	64	<	<	<	<
694	October, 2010	Mo	32	<	<	<	128	<	<	<	<	<	<	<
713	November, 2010	Nguyen Cong Tru	128	8	64	<	2048	512	<	<	<	<	<	<
733	November, 2010	Cau Dong	16	<	16	<	<	<	<	<	<	<	<	<
744	November, 2010	Phung Hung	32	<	16	<	1024	<	<	<	<	<	<	<
760	November, 2010	Kham Thien	64	16	8	<	512	512	<	128	<	<	<	<
761	November, 2010	Kim Lien	8	8	256	<	2048	1024	<	<	<	<	<	<
843	December, 2010	Kim Lien	<	64	<	<	<	<	<	16	<	<	<	<
892	December, 2010	Mo	32	128	64	16	<	<	<	<	<	<	<	<
946	January, 2011	Hom	8	<	16	8	<	<	<	<	16	<	<	8
956	January, 2011	Kham Thien	128	128	32	<	<	<	<	<	<	<	<	<
980	January, 2011	Bach Khoa	<	16	<	16	16	<	<	<	<	8	<	<
981	January, 2011	Mai Dong	<	8	128	<	<	<	<	<	<	<	<	<
1170	March, 2011	Kim Lien	16	64	16	<	64	<	<	<	<	<	<	<
1403	June, 2011	Hom	16	16	<	<	<	<	<	<	32	<	<	<
1414	June, 2011	Nguyen Cong Tru	<	<	<	<	<	<	<	<	32	<	<	8
1438	June, 2011	Ha Dong	32	<	8	<	<	<	<	16	<	<	<	<
1631	August, 2011	Ha Dong	<	32	16	<	<	<	<	<	<	<	<	<
1698	August, 2011	Kim Lien	<	<	<	32	8	<	<	<	<	<	<	<
2335	March, 2012	Ha Dong	1024	<	<	<	<	<	<	<	<	<	<	<
Control***	—	—	<	<	<	<	<	<	<	<	<	<	<	<

*, 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.

	Subtypes used for antigen					
	H3	H4	H6	H7	H9	H11
No. of positive samples	47 (1.98%)	6 (0.25%)	183 (7.7%)	0	10 (0.42%)	16 (0.67%)
No. of negative samples	2331	2372	2195	2378	2368	2362
No. of total samples	2378	2378	2378	2378	2378	2378

Table 3

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

		H5N1 clade 2.3.2.1		
		Positive	Negative	Total
H5N1 clade 2.3.4	Positive	265 (11.1%)	282 (11.9%)	547 (23%)
	Negative	247 (10.4%)	1584 (66.6%)	1831 (77%)
	Total	512 (21.5%)	1866 (78.5%)	2378