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Seroevidence for a High Prevalence of Subclinical Infection With Avian Influenza A(H5N1) Virus Among Workers in a Live-Poultry Market in Indonesia

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Background. In Indonesia, highly pathogenic avian influenza A(H5N1) virus has become endemic in poultry and has caused sporadic deadly infections in human. Since 2012, we have conducted fixed-point surveillance of avian influenza viruses at a live-poultry market in East Java, Indonesia. In this study, we examined the seroprevalence of avian influenza A(H5N1) virus infection among market workers.

Methods. Sera were collected from 101 workers in early 2014 and examined for antibody activity against avian A(H5N1) Eurasian lineage virus by a hemagglutination-inhibition (HI) assay.

Results. By the HI assay, 84% of the sera tested positive for antibody activity against the avian virus. Further analysis revealed that the average HI titer in 2014 was 2.9-fold higher than in 2012 and that seroconversion occurred in 44% of paired sera (11 of 25) between 2012 and 2014. A medical history survey was performed in 2016; responses to questionnaires indicated that none of workers had had severe acute respiratory illness during 2013.

Conclusions. This study provides evidence of a high prevalence of avian A(H5N1) virus infection in 2013 among workers at a live-poultry market. However, because no instances of hospitalizations were reported, we can conclude the virus did not manifest any clinical symptoms in workers.

Keywords. influenza virus; avian; H5N1; seroepidemiology; poultry; hemagglutination inhibition; HI; subclinical infection; seroconversion.

Influenza is one of the most common infectious diseases, affecting millions of people around the world every year. Occasionally, it causes a catastrophic pandemic such as the Spanish flu of 1918, which killed 30 million–50 million people worldwide. In Indonesia, highly pathogenic avian influenza A(H5N1) virus has been endemic in poultry since 2004 and has caused sporadic deadly infections in human. Potential adaptation for human-to-human transmission raises concerns about its pandemic potential. Furthermore, continuing occurrences of human infection may cause the emergence of a novel influenza virus through genetic reassortment during influenza virus coinfection.

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According to data published by the World Health Organization (WHO), between 2003 and 2015, the cumulative number of confirmed human cases of avian influenza A(H5N1) virus infection in humans totaled 842, with 447 resulting in death (fatality rate, 53%). Of these, 199 cases were reported in Indonesia, with 167 deaths (fatality rate, 84%) [1]. On the bases of these high fatality rates, the WHO and many countries have implemented emergency plans for possible scenarios of a pandemic caused by an emerging virus of avian influenza A(H5N1) origin. However, these fatality rates represent patients with severe acute respiratory illness caused by influenza A(H5N1) virus infection, and the actual fatality rate per infection is unknown. Moreover, the prevalence of asymptomatic/subclinical virus infections is not well understood; laboratory-confirmed cases are rare, and there may be numerous instances of undetected cases [2–4].

Live-poultry markets were shown to be significant locations for avian-to-human transmissions of avian influenza virus, and contact with sick or dead poultry was identified as a risk factor [5–8]. It is important to monitor live-poultry markets for coinfection with avian and human influenza viruses and consequent genetic reassortment between them. Since 2012, we have continued fixed-point surveillance for avian influenza viruses at a

live-poultry market in East Java, Indonesia, paying particular attention to transmission of avian influenza A(H5N1) viruses to the market workers. In this study, we tested for antibody against highly pathogenic avian influenza A(H5N1) viruses in serum samples from market workers.

METHODS

Samples From Market Workers

The live-poultry market where we collected blood and swab samples for this study receives livestock poultry (around 600–700 chickens and 100–200 ducks and Muscovy ducks daily) from surrounding farms and traditional backyards. This market provides a slaughtering service to customers. There are approximately 200 workers, consisting of live-poultry sellers, live-bird sellers, butchers, market-cleaning personnel/janitors, and other nearby stall owners. During 2012–2016, we invited workers to participate as volunteers in the surveillance study and seroepidemiologic analysis. A total of 63 market workers participated in this study in April 2012, 101 participated in February 2014, 100 participated in February 2015, and 142 participated in March 2016. Blood and oropharyngeal swab samples were collected from each participant. The swabs were assayed by reverse-transcription polymerase chain reaction (RT-PCR) for viral RNA and blood used for serologic assays. Swabs were eluted in 1 mL of 0.5% bovine serum albumin in Tris-buffered saline containing glucose [9] and stored at −80°C until use. Many participants were recruited for serial sampling, allowing us to collect paired sera. The aim of the study was communicated to all participants, who then provided written informed consent. Participants were also asked to fill in medical history questionnaires, with guidance provided by clinicians from our team. Institutional review boards at Airlangga University and Kobe University formally approved this study; the document identifiers are 181/EC/KEPK/FKUA/2014 and 1095, respectively.

RT-PCR

Detection of the viral genomes was performed with 1-step TaqMan real-time RT-PCR, using a QuantiTect Probe RT-PCR kit (Qiagen, Tokyo, Japan). Extraction of RNA from swabs was performed using a Qiamp MinElute Virus Spin Kit (Qiagen, Tokyo, Japan). The reaction mixture consisted of 5 µL of template RNA, each primer at a final concentration of 0.6 µM, 0.1 µM probe, and QuantiTect probe RT-PCR mix, and it was subjected to a 1-step assay with an ABI model 7300 instrument under the following conditions: step 1, reverse transcription for 30 minutes at 50°C; step 2, incubation for 15 minutes at 95°C to activate *Taq* polymerase; and step 3, 45 cycles for 15 seconds at 94°C and 75 seconds at 56°C. Primers and TaqMan probes were designed as described by the National Institute of Infectious Diseases, Japan [10, 11]. The primer set for subtype H5 hemagglutinin (forward, 5′-CGATC TAAAT GGAGT GAAGC CTC-3′; reverse, 5′-CCTTC TCTAC TATGT AAGAC CATTC-3′) detects the hemagglutinin gene of both Eurasian and Indonesian lineages in avian influenza A(H5N1) viruses. For differential detection, we made an additional reverse primer (5′-TCAAA ATGRT TTRTB CTGCT CA-3′); the set of the above forward primer and this reverse primer detects only the hemagglutinin gene of Eurasian lineage. To both sets, the following TaqMan probe was added: FAM-AGCCA TCCYG CTACA CTACA-MGB. For detection of the M gene of influenza A viruses, we used a set of primers (forward, 5′-CCMAG GTCGA AACGT AYGTT CTCTC TATC-3′; reverse, TGCAG RATYG GTCTT GTCTT TAGCC AYTCCA-3′) and probe (FAM-ATYTC GGCTT TGAGG GGGCC TG-MGB). This set detects all avian and human seasonal influenza A viruses tested in this study.

Isolation of Avian Influenza Viruses From Poultry

Our team's veterinarian observed the poultry for clinical signs of A(H5N1) infection: lethargy, shortened neck, retracted feather, diarrhea, torticollis, dyspnea, and clinical death. Cloacae swabs were obtained from sick or dead poultry at live-poultry markets, farms, and backyards in East Java. Swab samples were inoculated into the chorioallantoic cavity of 10-day-old embryonated chicken eggs and incubated for 2 days at 37°C. After the eggs were chilled at 4°C overnight, chorioallantoic fluids were harvested and tested for hemagglutination activity. Positive samples were then examined for avian influenza viral genome by RT-PCR.

Hemagglutination (HA) Assay

Serial 2-fold dilutions of virus samples were made in 50 µL of phosphate-buffered saline (PBS) in 96-well, U-bottomed plates. To each well, 50 µL of 0.5% (v/v) chicken red blood cells (RBCs) was added. After incubation for 1 hour at 4°C, HA patterns were read. The HA titers were determined from the last dilutions showing complete HA and expressed by the reciprocal of the dilutions.

Hemagglutination-Inhibition (HI) Assay

The HI assay to detect specific antibody activity was performed following the Salk pattern method [12] after adjusting volumes to 96-well microplates. The sera were treated with 3 volumes of receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) at 37°C overnight. The enzyme was inactivated by incubation for 30 minutes at 56°C, and then nonspecific hemagglutinating substances existing in serum were removed by adsorption with one-fourth volume of chicken RBC pack. Serial 2-fold dilutions of the treated sera were made in 25 µL of PBS in 96-well, U-bottomed plates, and a 25-µL aliquot of indicator viruses freshly prepared to have an HA titer of 8 in PBS was added to each well. After the serum and virus mixture was incubated for 60 minutes at room temperature, 50 µL of 0.5% chicken RBCs was added. Plates were incubated at 4°C and read after 60 minutes. The HI titer was expressed as the reciprocal of the highest dilution of serum in which HA was completely inhibited. In this

study, when a transit pattern occurred between complete inhibition and negative inhibition wells, the highest dilution was estimated as being midway between the 2-fold dilutions. The HI assay was repeated more than twice. The paired sera collected at different times were tested in the same assay run. For non-paired sera, owing to the large number of samples, we were not able to perform simultaneous assays. Thus, when testing, we overlapped the samples; 12 or 24 samples from the previous assay were tested again in the subsequent assay. Because we did not have WHO-validated serum specific for H5 clade 2.3.2.1 hemagglutinin, we used pooled sera with high HI titers as positive. For statistical evaluation, the mean value, standard deviation (SD), and standard error (SE) were calculated for the $\log_2$ HI titer.

Indicator Viruses

As an avian influenza A(H5N1) indicator virus in HI assays, we used one of our highly pathogenic A(H5N1) isolates from turkey: influenza A/turkey/East Java/Av154/2013(H5N1) virus Eurasian lineage (hereafter, "Av154[H5N1 Eur]"; unpublished data; Table 1). The virus was isolated from an outbreak in September 2013 at a turkey farm in East Java; approximately 150 turkeys suddenly died with or without apparent clinical symptoms within 5 days. The isolate belongs to hemagglutinin clade 2.3.2.1 of the Eurasian lineage, which had not been found in Indonesia until December in 2012 [13]. The amino acid sequence at the cleavage site of the hemagglutinin gene indicates that this virus is highly pathogenic. For evaluation of the specificity, 2 additional viruses from our isolates shown in Table 1 were used as indicators: influenza A/chicken/East Java/Av240/2014(H5N1) virus of hemagglutinin clade 2.1.3.3 Indonesian lineage (hereafter, "Av240[H5N1 Ind]") and influenza A/duck/East Java/Av39/2013(H3N6) virus (hereafter, Av39[H3N6]"; unpublished data). All tests involving live A(H5N1) viruses were conducted in a biosafety level 3 laboratory at the Institute of Tropical Disease, Airlangga University. As indicator viruses for seasonal human influenza viruses, we used influenza A/Sydney/5/1997(H3N2) virus (hereafter, "Syd[H3N2]") and 2009 pandemic A/East Java/D264/2015(H1N1) virus (hereafter, "D264[H1N1]"), which we isolated from a patient with influenza-like illness in East Java in 2015 (unpublished data).

RESULTS

Isolation of Avian Influenza Viruses Circulating in Poultry in East Java

Table 1 summarizes isolation of avian influenza viruses from sick or dead poultry 6 months before and at the same time as the collection of samples from market workers. The upper half of the table shows results for samples collected in and around the market during May–September 2013 (dry season). Fifteen were identified as A(H5N1) Eurasian lineage viruses and 1 as A(H3N6) virus by RT-PCR, followed by sequencing. Avian A(H5N1) Indonesian lineage virus was not detected. A(H5N1) virus was isolated from 0.10% of poultry observed. The lower half of the table shows results from samples collected during January–February 2014 (rainy season) at the market site. Sixteen were identified as A(H5N1) Eurasian lineage viruses, 6 as Indonesian lineage viruses, and 1 as A(H3N6) virus. A(H5N1) virus was isolated from 0.19% of poultry during the 2014 rainy season, or about 2 times the percentage during the 2013 dry season.

Detection of Influenza Viruses in Swab Samples From Live-Poultry Market Workers

Because of close and frequent contact with A(H5N1)-positive poultry, we hypothesized that market workers might be implicated in avian influenza virus infection. We therefore examined oropharyngeal swab specimens from workers for the presence

Table 1. Influenza A Virus Isolation From Poultry in East Java, Indonesia

Poultry	Population Observed	Samples Collected	HA-Positive Eggs Harvested	Subtype/Lineage, by RT-PCR		
				H5/Eur	H5/Ind	H3
May–Sep 2013[a]						
Ducks, no.	13 000	56	9	5	0	1
Muscovy ducks, no.	400	22	6	5	0	0
Chickens, no.	1800	42	10	3	0	0
Turkeys, no.	300	2	2	2	0	0
Subtotal, no. (%)	15 500 (100)	122 (0.787)	27 (0.174)	15 (0.097)	0 (0)	1 (0.006)
Jan–Feb 2014[b]						
Ducks, no.	1800	13	7	3	1	1
Muscovy ducks, no.	720	9	2	1	1	0
Chickens, no.	9000	82	46	12	4	0
Subtotal, no. (%)	11 520 (100)	104 (0.903)	55 (0.477)	16 (0.139)	6 (0.052)	1 (0.009)
Overall, no. (%)	27 020 (100)	226 (0.836)	82 (0.303)	31 (0.115)	6 (0.022)	2 (0.007)

Abbreviations: Eur, Eurasian lineage; HA, hemagglutination; Ind, Indonesian lineage; RT-PCR, reverse-transcription polymerase chain reaction.

[a] Samples were collected from sick or dead poultry in live-poultry markets, farms, and backyards in East Java.

[b] Samples were collected from sick or dead poultry in a live-poultry market in East Java.

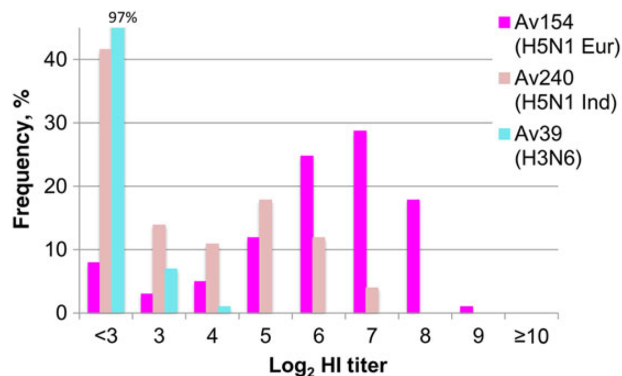

Figure 1. Distributions of hemagglutination-inhibition (HI) titers against avian influenza type A viruses among live-poultry market workers. Sera collected from 101 workers in February 2014 were examined by HI assays for antibody activity against influenza A/turkey/East Java/Av154/2013(H5N1) virus of H5 clade 2.3.2.1 Eurasian lineage (Av154[H5N1 Eur]), influenza A/chicken/East Java/Av240/2014(H5N1) virus of H5 clade 2.1.3.3 Indonesian lineage (Av240(H5N1 Ind]), and influenza A/duck/East Java/Av39/2013(H3N6) virus (Av39[H3N6]).

of the viral genome by RT-PCR. Avian A(H5N1) virus was not detected in any of the swab specimens collected from 101 workers in February 2014. Seasonal human A(H3N2) virus was detected in 3 swab specimens and 2009 pandemic A(H1N1) virus in 1.

Prevalence of Antibody Against Av154(H5N1 Eur) Virus Among Market Workers

For serological detection of infection, we subjected sera collected from the workers to an HI assay to detect antibody against Av154(H5N1 Eur) virus. As shown in Figure 1 and Table 2, 84% of 101 serum samples were positive when HI titers of ≥32 (ie, 2^5) were judged to be positive. The geometric mean HI titer was 84 (ie, $2^{6.4}$), and the median was 111 (ie, $2^{6.8}$). The rate of detection was 62% according to the WHO criterion (ie, an HI titer of ≥80) [14]. Specificity of the antibody activity was examined by additional HI assays, using Av240(H5N1 Ind) and Av39 (H3N6) as avian influenza A virus indicators. For antibody activity against Av240(H5N1 Ind), 34% of sera tested positive, and the geometric mean titer was 16 (ie, 2^4). Antibody against Av39 (H3N6) was not detected at a positive level. These results indicated that the HI assay had a high specificity for antibody against Av154(H5N1 Eur) virus. As lower risk populations, we examined sera obtained from people other than market workers: 28 serum samples were collected during 2001–2011 from healthy adults living in Japan, 50 samples were collected in November 2015 from healthy volunteers at a blood bank in East Java, and 43 samples were collected in November 2013 from medical staff at a hospital in East Java. Figure 2 compares the distribution of HI titers against Av154(H5N1 Eur) virus among 3 control groups and market workers. While 20 serum samples (40%) from the hospital medical staff tested positive (Figure 2*C*), none of the sera from the other 2 groups possessed antibody at a positive level (Figure 2*A* and 2*B*). None of the market or healthcare workers had previously participated in H5 vaccine studies.

As for antibody against seasonal Syd(H3N2) virus, around 80% of all of the 4 groups tested positive. As shown in Figure 3, correlation between HI titers against Av154(H5N1 Eur) virus

Table 2. Seroconversion of Hemagglutination-Inhibition (HI) Titers Against Influenza A/Turkey/East Java/Av154/2013(H5N1) H5 Clade 2.3.2.1 Eurasian Lineage Virus (Av154[H5N1 Eur]) Among Live-Poultry Market Workers

		Log_2 HI Titer Against Av154(H5N1 Eur)		*P*[a] vs		Positive, No. (%)[d]		
Study Period, Analysis Group	Subjects, No.	Mean (Median) ± SD[a]	Ratio to Feb 2014 Titer, All	Feb 2014 Titer, Pair[b]	Feb 2014 Titer, All[c]	≥32	≥80	Seroconversion,[e] No. (%)
Apr 2012								
All	63	4.9 (4.9) ± 1.17	0.35		<.0001	30 (48)	8 (13)	. . .
Paired with Feb 2014	25	4.8 (4.8) ± 1.25	0.32	.0028		11 (44)	2 (8)	. . .
Feb 2014								
All	101	6.4 (6.8) ± 1.76	1.00			85 (84)	63 (62)	. . .
Paired with Apr 2012	25	6.0 (6.5) ± 2.12	0.74			19 (76)	14 (56)	11 (44)
Paired with Feb 2015	36	6.3 (6.5) ± 1.68	0.97			30 (83)	21 (58)	. . .
Paired with Mar 2016	58	6.2 (6.8) ± 1.39	0.98			47 (81)	36 (62)	. . .
Feb 2015								
All	100	5.3 (5.3) ± 1.34	0.46		<.0001	59 (59)	22 (22)	. . .
Paired with Feb 2014	36	5.3 (5.3) ± 1.44	0.48	.0002		22 (61)	9 (25)	1 (3)
Mar 2016								
All	142	4.0 (4.1) ± 1.45	0.19		<.0001	37 (26)	12 (8)	. . .
Paired with Feb 2014	58	4.1 (4.3) ± 1.39	0.20	<.0001		15 (26)	5 (9)	3 (5)

[a] For the calculation, 4 was assigned when the HI titer was below the minimal detection level of 8.

[b] By the Wilcoxon signed ranks test of log_2 HI titers.

[c] By the Mann–Whitney *U* test of log_2 HI titers.

[d] Titers of ≥32 were considered positive in this study, whereas titers of ≥80 are considered positive by the World Health Organization.

[e] Defined as a ≥4-fold increased titer.

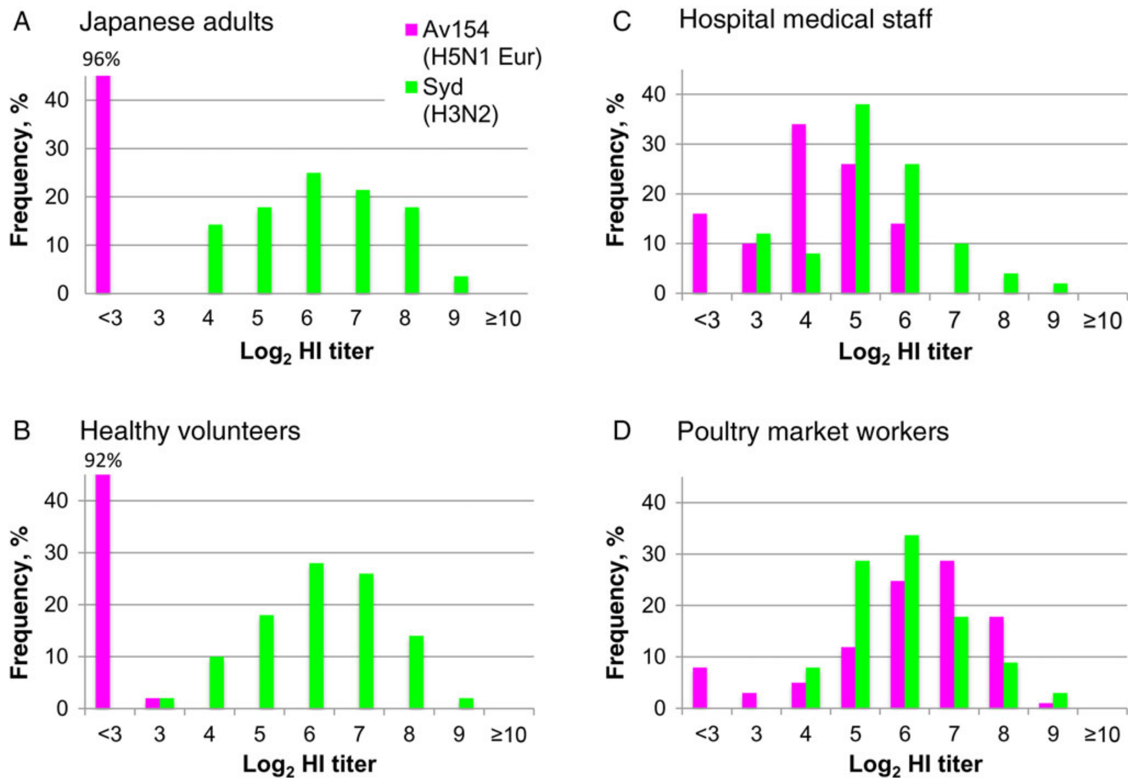

Figure 2. The distributions of hemagglutination-inhibition (HI) titers against influenza A/turkey/East Java/Av154/2013(H5N1) virus Eurasian lineage (Av154[H5N1 Eur]) and seasonal influenza A/Sydney/5/1997(H3N2) virus (Syd[H3N2]) were compared among 4 groups: 3 control groups and 1 group of market workers. Sera were collected during 2001–2011 from 28 healthy adults living in Japan (*A*), in November 2015 from 50 healthy volunteers at a blood bank in East Java (*B*), in November 2013 from 43 medical staff of a hospital in East Java (*C*), and in February 2014 from 101 workers at a live-poultry market in East Java (*D*).

and those against seasonal Syd(H3N2) and D264(H1N1) viruses was analyzed by scattered plotting, and the Pearson product-moment correlation coefficient, *r*, was calculated. The *r* was 0.0733 (95% confidence interval [CI], −.1238–.2650; $P = .4659$) for Av154(H5N1 Eur) versus Syd(H3N2) and 0.1649 (95% CI, −.0315–.3491; $P = .0993$) for Av154(H5N1 Eur) versus D264(H1N1). These correlations were not significant level in a 95% significance test, indicating that antibody activity against Av154(H5N1 Eur) virus was not derived from cross-reactivity with these human seasonal viruses.

Seroconversion of HI Titers Against Av154(H5N1 Eur) Virus During 2012–2016

To investigate seroconversion, we measured HI titers of sera collected during 2012–2016 from market workers. As shown in Table 2, the average HI titer against Av154(H5N1 Eur) in February 2014 was 2.9-fold that in April 2012 (1.00/0.35), and in February 2015 and March 2016, titers decreased to 0.46-fold (0.46/1.00) and 0.19-fold (0.19/1.00) that in February 2014, respectively. The percentage of workers with seroconversion (defined as a ≥4-fold increase in titer), based on paired sera analysis, was 44% (11 of 25) in 2014, 3% (1 of 36) in 2015, and 4% (3 of 58) in 2016. Figure 4 compares the kinetics of average HI titers against Av154(H5N1 Eur), Av240(H5N1 Ind), human seasonal Syd(H3N2), and D264(H1N1) viruses between 2012 and 2016. The kinetics of HI titers against Av154(H5N1 Eur) were quite different from those against Syd(H3N2) and D264(H1N1); there was a clear peak in 2014, increasing approximately 3-fold from 2012 and decreasing to 0.5-fold in 2015 and 0.2-fold in 2016. In contrast, no significant peaks were observed for seasonal influenza viruses. The kinetics pattern of HI titers against Av240(H5N1 Ind) virus was somewhat similar to that against Av154(H5N1 Eur), but the titer at the peak was about 0.20. Table 3 shows the kinetics of HI titers against Av154 (H5N1 Eur) in 13 workers who participated continuously between 2012 and 2016. The kinetics of the average HI titer for these 13 workers was similar to that of all participants. There were 6 positive seroconversions from 2012 to 2014, and 6 negative seroconversions (≤25% decrease) from 2014 to 2016. On the contrary, the kinetics of HI titers against seasonal Syd (H3N2) virus was flat, and there were no seroconversions. The results obtained by the present HI assays reveal that Av154(H5N1 Eur) virus had previously infected workers at the live-poultry market. Since avian A(H5N1) of hemagglutinin clade 2.3.2.1 of Eurasian lineage virus was newly introduced to Indonesia in late 2012, antibody-positive workers had possibly been infected with the virus during 2013.

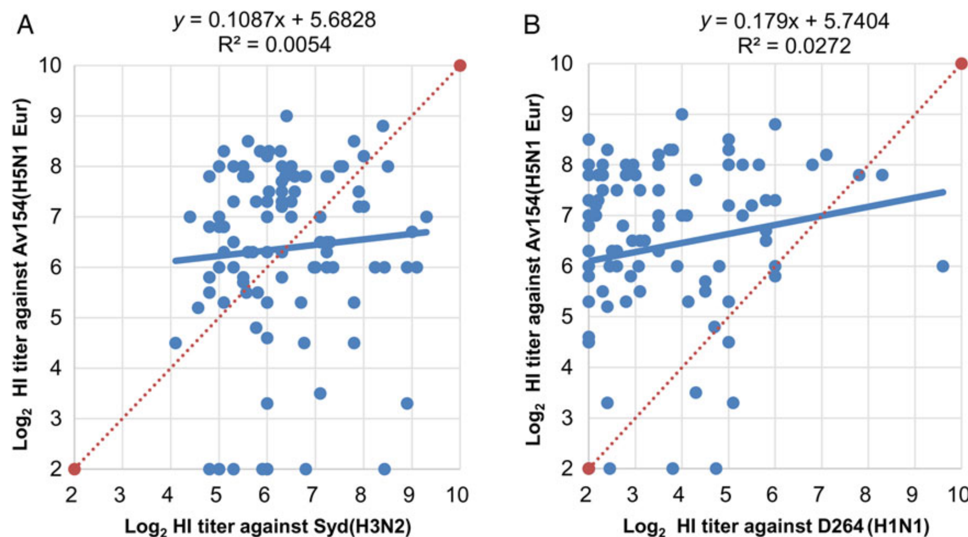

Figure 3. No correlation of hemagglutination-inhibition (HI) titers against influenza A/turkey/East Java/Av154/2013(H5N1) virus Eurasian lineage (Av154[H5N1 Eur]) with those of seasonal influenza A/Sydney/5/1997(H3N2) virus (Syd[H3N2]) and 2009 pandemic influenza A/East Java/D264/2015(H1N1) virus (D264[H1N1]). Sera were collected in February 2014 from 101 workers at a live-poultry market in East Java. Correlations of HI titers against Av154(H5N1 Eur) virus (vertical axis) with those against Syd(H3N2) virus (*A*) and D264(H1N1) (*B*; horizontal axis) were analyzed. The Pearson product-moment correlation coefficient, *r*, was calculated, with values of 0.0733 (95% confidence interval [CI], −.1238–.2650; $P = .4659$) for Av154(H5N1 Eur) vs Syd(H3N2) virus (*A*) and 0.1649 (95% CI, −.0315–.3491; $P = .0993$) for Av154(H5N1 Eur) vs D264(H1N1). These correlations were not significant level in a 95% significance test, indicating that antibody activity against Av154(H5N1 Eur) virus was not derived from cross-reactivity with these human seasonal viruses.

Clinical Surveillance for Severe Acute Respiratory Illness Among Market Workers

In the 2016 study, we conducted a survey of medical histories by giving questionnaires to the 58 workers who had also participated in the 2014 study. None of the participants reported histories of severe acute respiratory illness or hospitalization for any illness in 2013, although 47 (81%) were positive for antibody against Av154(H5N1 Eur) virus, based on analysis of 2014 sera by an HI assay (Table 2). Thirty of 47 workers with positive HI assay findings had influenza-like illness during 2013. The most commonly reported symptoms were cough, rhinorrhea, and fever, followed by headache and diarrhea. Five of 11 workers with negative HI assay findings also had influenza-like illness. The *P* value (by the Fisher exact test) for the correlation between HI assay positivity and influenza-like illness was .2621 and nonsignificant.

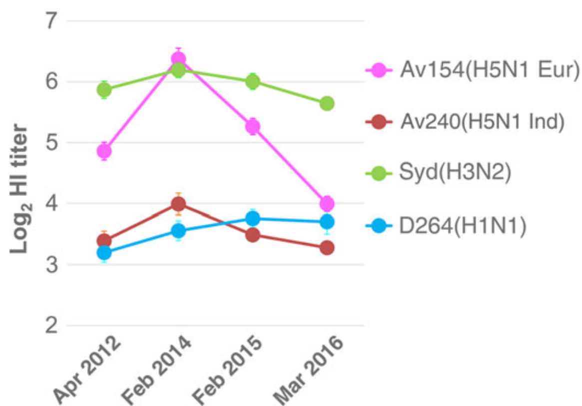

Figure 4. Kinetics of hemagglutination-inhibition (HI) titers against avian and seasonal human influenza A viruses. There were 406 serum samples evaluated by the HI assays; 63 were from April 2012, 101 were from February 2014, 100 were from February 2015, and 142 were from March 2016 (Table 2). Among them, there were 13 workers who participated continuously between 2012 and 2016 (Table 2). The average HI titers against influenza A/turkey/East Java/Av154/2013(H5N1) virus Eurasian lineage (Av154[H5N1 Eur]), influenza A/chicken/East Java/Av240/2014(H5N1) virus Indonesian lineage (Av240[H5N1 Ind]), influenza A/Sydney/5/1997(H3N2) virus (Syd [H3N2]), and 2009 pandemic influenza A/East Java/D264/2015(H1N1) virus (D264 [H1N1]) during 2012–2016 were plotted with standard errors, and the kinetics were compared.

DISCUSSION

It has been reported elsewhere that the seroprevalence of antibodies against avian influenza A(H5N1) virus among poultry workers was 0%–10% by microneutralization assays [15–24]. Results of this study show that sera collected from 84% of 101 workers in 2014 at a live-poultry market in East Java tested positive against Av154(H5N1 Eur) virus by the HI assay. The specificity of the HI assays against Av(H5N1 Eur) virus was demonstrated. The HI antibody specifically inhibited Av154 (H5N1 Eur) virus, as shown in Figure 1. Correlation analysis revealed that the antibody activity against Av154(H5N1 Eur) virus was not derived from cross-reactivity with human seasonal A(H3N2) and 2009 pandemic A(H1N1) viruses (Figure 3). The distribution pattern of HI titers of low-risk control groups also supported the specificity (Figure 2). Most importantly, the

Table 3. Seroconversion Against Influenza A/Turkey/East Java/Av154/2013(H5N1) H5 Clade 2.3.2.1 Eurasian Lineage Virus (Av154[H5N1 Eur]) in 13 Participants Present in All Study Periods

	Log$_2$ HI Titer, Mean±SE, Against							
	Av154(H5N1 Eur)				Syd(H3N2)			
Participant	Apr 2012	Feb 2014	Feb 2015	Mar 2016	Apr 2012	Feb 2014	Feb 2015	Mar 2016
L01	5.7 ± 0.35	**8.3 ± 0.10**	7.1 ± 0.27	*5.8 ± 0.20*	3.8 ± 0.20	4.3 ± 0.25	4.2 ± 0.20	3.3 ± 0.81
L09	5.0 ± 0.35	**8.3 ± 0.10**	*6.1 ± 0.27*	*3.5 ± 0.10*	4.5 ± 0.51	5.5 ± 0.42	4.1 ± 0.07	4.0 ± 0.14
L25	5.3 ± 0.3	**7.3 ± 0.1**	*3.4 ± 0.43*	*2.0 ± 0.1*	5.1 ± 0.51	4.8 ± 0.67	4.1 ± 0.07	4.1 ± 0.36
L42	6.0 ± 0	*2.0 ± 1.21*	**5.4 ± 0.19**	4.3 ± 0.3	4.8 ± 0.81	5.0 ± 0	4.4 ± 0.2	4.4 ± 0.25
L43	4.5 ± 0.51	5.3 ± 0.91	*3.0 ± 0.53*	3.5 ± 0.1	5.6 ± 0	6.0 ± 0	5.4 ± 0.2	4.1 ± 1.28
L44	2.8 ± 0.51	**6.0 ± 0.61**	5.6 ± 0.35	4.3 ± 0.3	6.8 ± 0.2	7.0 ± 0	6.9 ± 0.14	6.3 ± 0.22
L46	6.2 ± 0.15	6.5 ± 0.71	7.0 ± 0.57	5.3 ± 0.3	5.1 ± 0.51	6.6 ± 0	5.9 ± 0.14	5.2 ± 0.25
L49	2.3 ± 0.71	3.3 ± 0.1	3.6 ± 0.35	2.8 ± 1.21	7.3 ± 0	9.0 ± 0	7.9 ± 0.14	7.2 ± 0.25
L67	6.6 ± 0	7.8 ± 0.61	7.0 ± 0.57	*5.1 ± 0.1*	8.0 ± 0	7.0 ± 0	6.9 ± 0.14	6.3 ± 0.3
L77	4.5 ± 0.51	**6.8 ± 0.4**	5.6 ± 0.03	*4.1 ± 0.1*	4.0 ± 1.01	4.1 ± 0.42	3.6 ± 0.51	3.1 ± 0.66
L78	6.0 ± 1.01	7.8 ± 0.4	7.4 ± 0.57	6.0 ± 0.61	6.1 ± 0.51	6.1 ± 0.59	5.8 ± 0.12	5.2 ± 0.25
L80	4.7 ± 0.66	**7.5 ± 0.91**	*4.4 ± 0.75*	*3.5 ± 0.1*	4.3 ± 0.71	5.5 ± 0.42	3.7 ± 0.38	*3.1 ± 0.36*
L83	4.4 ± 0.4	5.2 ± 0.4	5.1 ± 0.33	3.3 ± 0.1	3.8 ± 0.2	3.5 ± 0.42	3.6 ± 0.35	3.6 ± 1

Conversion to seropositivity (ie, a ≥4-fold [2 log$_2$] increase from April 2012 or February 2014) is in bold, and conversion to seronegativity (ie, a ≤0.25-fold decrease from February 2014 or February 2015) is in italics.

Abbreviations: HI, hemagglutination inhibition; SE, standard error; Syd(H3N2), influenza A/Sydney/5/1997(H3N2) virus.

average HI titer in 2014 was higher than in 2012, and seroconversion was observed in 44% of paired sera between 2012 and 2014 (Table 2). On the basis of these results, we concluded that the antibody activity shown by the HI assays was specific to Av154(H5N1 Eur) virus infection and that it was not derived from nonspecific inhibitory substances existing in sera.

In this study, we defined an HI titer of ≥32 as positive, based on the HI titer distributions obtained for the market workers (Figure 1) and the control groups (Figure 2). When HI titers of ≥80 were, in accordance with the WHO recommendation [14], considered positive, 62% of sera collected in 2014 were positive for antibody against Av154(H5N1 Eur) virus (Table 2). However, the strict WHO criterion is primarily for definitive diagnosis of A(H5N1) infection in suspected human cases. We think that a low cutoff is worth considering for asymptomatic infections. For example, detection of antibody against Av154 (H5N1 Eur) virus among hospital medical staff was 40% by our criterion, while it was 4% by the WHO criterion (Figure 2). A frequency of 40% seems possible for the medical staff, considering that the frequency in the other 2 control groups was 0% by both criteria. We should not ignore possibility of human-to-human transmissions within the hospital. The high percentage of influenza virus positivity in our study was probably due to (1) the use of a local circulating virus that had not been detected in Indonesia until December in 2012 as an indicator [13] and (2) the collection of sera ≤1 year after possible first exposure to the virus.

We showed in Table 2 the increase in the average HI titer against Av154(H5N1 Eur) in 2014 and seroconversion in paired sera from 2012 and 2014. This presented strong evidence of influenza virus infection between 2012 and 2014. Buchy et al reported that A(H5N1) antibody titers in asymptomatic infection were lower and decreased to levels below the threshold of positivity within 1 year [25]. In this study, we also observed a rapid decrease of HI titers; of 13 workers who participated in all 4 studies, 6 converted to seronegativity between 2014 and 2016, while 6 converted to seropositivity between 2012 and 2014. We propose that conversion not only to seropositivity but also to seronegativity should be considered evidence of infection. It is important to know the kinetics of antibodies, and a low cutoff is worth considering for interpretation of seroepidemiologic studies.

To validate our HI assay results, we have been following up with microneutralization assays of sera collected from poultry market workers. The results of our microneutralization assays also indicated a high prevalence of avian Av154(H5N1 Eur) virus infection. However, the Pearson correlation revealed significant correlation ($r = 0.4174$; 95% CI, .2357–.5709; $P < .0001$) between the microneutralization titers against Av154(H5N1 Eur) virus and those against seasonal D264 (H1N1) virus, suggesting the cross-neutralization activity of antibodies to these viruses. In contrast, such significant correlation was not observed between HI titers associated with these viruses, as shown in Figure 3. Thus, we are beginning to think that the HI assay may be more suitable for detecting antibodies against specific subtypes of avian A(H5N1) virus. Further investigations into the characteristics of antibodies against A(H5N1 Eur) virus are needed.

Since seasonal influenza A(H3N2) and 2009 pandemic A(H1N1) viruses were detected by RT-PCR in the swab samples

collected in 2014 from the market workers, there could have been opportunity for coinfection by avian and human influenza viruses in the market during 2013–2014. Monitoring for coinfection at live-poultry markets is imperative. Our study provides seroevidence for the high prevalence of influenza A(H5N1) virus infection among live-poultry market workers in East Java in 2013. Sera from 84% of the workers tested positive for antibody activity against Av154(H5N1 Eur) virus. Medical history questionnaires reported no hospitalizations for severe acute respiratory illness during 2013. Although a significant percentage of the workers had had influenza-like illness, it could not be related to Av154(H5N1 Eur) virus infection. We therefore conclude that Av154(H5N1 Eur) virus did not cause clinical symptoms in humans, suggesting that the highly pathogenic nature of avian influenza A(H5N1) viruses was not inherited when transmitted to humans.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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