Seroprevalence of Avian Influenza virus subtype H5N1 in chickens of the two main poultry market in Surakarta area, Central Java

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Abstract. Avian influenza (AI) subtype H5N1 virus is very contagious among domestic poultry such as chicken (Gallus gallus) and potentially infecting humans through direct or indirect contact with the infected animal. In the Surakarta area, Central Java, there are two major poultry markets, Silir poultry market and Jumantono farm. Here, we determined the seroprevalence of the AI virus in chickens sold in both poultry markets. The method was conducted using an explorative experiment by collecting 140 samples of chicken blood serum. The presence of antibodies to the AI virus subtype H5N1 was measured through a standard serological test via hemagglutination-inhibition (HI) assay. The HI assay detected a large antibody difference against AI from samples of both locations; 4.28% from Silir poultry market and 48.57% from Jumantono farm. Because the chickens from the Silir poultry market were not vaccinated, the level of antibody detected indicated that some chickens were exposed to the virus through infection. Meanwhile, the high positivity rate in Jumantono farm might reflect the presence of neutralizing antibody, since the chickens from this farm received periodic vaccinations. Continuous quality surveillance in the environment of the poultry market is essential to reduce the impact of AI outbreaks against chickens and humans.

1. Introduction
Avian influenza (AI) subtype H5N1 is considered highly pathogenic and deadly to poultry. This disease is considered endemic in six Asian countries, including Indonesia. Chicken infected by AI showed clinical symptoms, such as head and neck edema, torticollis, also bluish-purple hue in combs and waddles (cyanosis). The mortality rate in infected chicken and duck reached ~30%, even after being vaccinated [1]. The spread of AI in humans is relatively rare; however, positive cases showed that this disease is quite severe and can cause death [2]. World Health Organization (WHO) reported 288 human AI cases in Southeast Asia from early 2004 to 31 December 2013, with a case fatality rate (CFR) of 79.4% (181 cases). In April 2014, confirmed AI human cases in Indonesia reached 195 cases with 163 deaths [3]. Central Java province showed the highest mortality rate in human AI from 2005 to 2012 with a CFR of 92.3% or 12 deaths from 13 cases [4].

The transmittance of AI occurs through direct contact between infected poultry with healthy poultry. Infected poultries release viruses from their respiratory system, conjunctiva, and feces. Besides that, indirect transmittance can also happen through air, foods, drinks, farming equipment,
animal cage, clothes, vehicles, egg crates, egg trays, which are contaminated with the AI virus [5]. AI can also infect humans from direct contact with infected and sick poultry [6].

A well-known method to identify AI is by hemagglutination-inhibition (HI) test. The hemagglutinin protein (HA) can bind to the red blood cell receptor and caused clumping (hemagglutination). In serum containing specific antibodies for AI, the interaction between antibodies and antigenic sites on hemagglutinin molecules will inhibit the hemagglutination process. The inhibition of hemagglutination reaction becomes the standard surveillance test's base to various subtypes of AI [7].

The poultry markets and farms have an essential role in propagating and spreading the AI virus between poultries and poultry to humans [1,8]. Silir poultry market is Surakarta city’s only poultry market established in 1978. The market area is 11,220 m² with 320 stalls and 27 poultry butcher stalls. Besides the market, the farm is also the most extensive AI virus clustering area. Jumantono poultry farm in Karanganyar Regency, Central Java, has been raising various chicken breeds, including domesticated chicken and free-range chicken (ayam kampung in Bahasa). This farm is managed by individuals and farmer groups that used the traditional method in raising poultries (i.e., chickens, duck). Chickens from the Jumantono farm were sold as layers (egg-laying chickens) and broilers (chickens raised for meat). The number of chickens in poultry farming in this district has increased during 2018-2019 [9], meaning that the management needs specific attention.

Based on those mentioned above, proper biosecurity and management in the poultry market and farming (i.e., poultry agriculture) is needed to prevent the AI epidemic. This study is a part of a sustainable monitoring effort on the environment vulnerable to widespread AI. This study investigates the seroprevalence of AI subtype H5N1 in chickens sold at Silir poultry market and Jumantono farm. These two locations were selected since they are the main locations where chickens consumed by citizens of Surakarta, Central Java, were sold.

2. Materials and methods

2.1. Blood sampling

Samples (140 blood samples) from the Silir poultry market and Jumantono farm were collected in September 2019. Chicken blood was obtained using disposable spuit through the chickens’ vena brachialis. Blood samples were inserted in the venoject tubes containing EDTA anticoagulant. The samples were immediately stored inside an icebox and transported to the laboratory. The chicken blood serum was obtained by separating serum and blood cells using a centrifuge. Unchecked samples were kept inside the refrigerator at 4°C for a maximum of four days [7].

2.2. Preparation of 1% erythrocyte suspension

Blood samples were collected from healthy chickens. In this study, SPF-free erythrocyte samples were collected from three chickens that were supervised and controlled by Surakarta’s Animal Health Laboratory. Blood samples collected were gently shaken to prevent clumping and lysis. Preparation of 1% erythrocyte suspension was done by taking 1mL chicken erythrocyte added with 99 mL phosphate buffered saline (PBS) with pH 7.2-7.4. The 1% erythrocyte suspension can be directly used or stored at 4°C.

2.3. Preparation of antigen 4 HA unit

Before the hemagglutination inhibition (HI) assay was done, the antigen used need to be confirmed as antigen 4 HA unit. A serological test was done, and HI was considered positive when antibody titer ≥ 2⁴ or log 2⁴ with antigen 4 HAU [10]. Therefore, the antigen needs to be converted into 4 HA unit. Antigen 4 HA unit was prepared with antibody titer on HA divided by 4 or reduced by 2 log.
hemagglutination inhibition (HI) assay

The hemagglutination inhibition (HI) assay was done by filling well on 1-10 microplate holes with 25 μl PBS using a multi-channel micropipette. Next, 25 μl chicken blood serum was added to hole number 1 and gradually diluted to hole number 10 by homogenization using the same pipette. Antigen AI4 HA unit of 25 μl was added to those ten microplate holes. Samples were shaken to homogenized them and incubated for ±15 minutes at room temperature (20-25˚C). Next, 25 μl of 1% blood cells were added to each microplate hole, and shaken to homogenize, then incubated again for ±30 minutes at room temperature. The HI titer was read by tilting the microplate and observing precipitate formed [10]. The HI titer is the highest serum dilution that causes the 4 HA unit complete inhibition [7].

Determination of seroprevalence avian influenza virus

Prevalence refers to the individual proportion in the population infected with disease or other conditions in a certain period. Positive seroprevalence AI virus subtype H5N1 was calculated with:

\[
P = \frac{\sum A}{\sum B} \times 100\%
\]

where:
A = The number of total AI positive samples
B = The total number of tested samples

3. Results and discussion

Free-range chicken (ayam kampung) from Silir poultry market did not receive any vaccines prior to this study. The results of hemagglutination inhibition showed that 3 out of 70 serum samples showed positive reactions (Table 1). These positive results indicate the presence of serum antibodies towards the AI H5N1 virus. The antibody formed as a natural response to viral infection because all sampled chickens have never been vaccinated before. The antibody titer produced is considered high or protective since it was detected between ≥ 4 log 2/log 24.

Table 1. The chicken antibody titer at silir poultry market and Jumantono Farm

| Location            | N  | 0   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|---------------------|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Silir poultry market| 70 | -   | -   | -   | -   | 2   | 1   | -   | -   | -   | -   | -   | -   |
| Jumantono farm      | 70 | 36  | 4   | 5   | 14  | 4   | 5   | 2   | -   | -   | -   | -   | -   |
| Total               | 140|     |     |     |     |     |     |     |     |     |     |     |     |

Contrary to the chickens in the Silir poultry market, chickens from Jumantono farm received repeated AI vaccines. However, our results showed only 34 samples (48%) from this farm containing positive antibody titer towards AI (Table 1). Among samples that showed positive results, only 11 had protective antibody titer levels (≥ 4 log 2/log 24). Moreover, 36 blood serum samples (52%) from Jumantono farm without antibody titer (20) to AI virus. Thus, a review is needed to check whether the vaccine given has successfully triggered antibody titer formation. The AI vaccine failure in poultry can be caused by the shift of virus variants available in the field, the use of unlicensed virus strain in the vaccine production, and the amount of antigen in vaccine inadequate to induce protective antibodies [11].

The level of antibody titer indicates the animal immunity to infection. The serological test (Table 2) showed that seroprevalence of AI virus in ayam kampung at Silir poultry market was 3 (4.28%) with a high/protective level to AI H5N1 virus. Samples from the Jumantono farm showed 34 (48.57%) samples were positively containing antibody titer, which is high enough to fight AI H5N1 virus. The duration of chicken’s active antibody titer in a protective level that resulted from vaccination or infection is influenced by several factors, e.g., disease challenges in the field, stress, and immunosuppressive condition [12]. Vaccinated poultry or poultry infected by the virus in the field will...
form antibodies (IgA, IgG dan IgM), which can be hereditied to their offspring [13]. IgG is the primary antibody in the blood serum. This antibody can be detected as soon as seven days post vaccinated/infected. A monthly serological test will help describe the protective antibody titer in chickens and estimate when re-vaccination should be done.

The poultry market can potentially spread the AI H5N1 virus because close contacts between poultry became possible since all kinds of poultry gathered in one place [14]. Our field observation showed three chickens from Silir poultry market with their blood serum containing antibody titer, although not showing viral symptoms (asymptomatic). Nevertheless, these infected chickens can be carriers of the AI virus and spread the viral infection to other poultry. It is possible that AI clinical symptoms did not appear in poultry exposed to a repeated, low amount of virus. In this case, the antibody anti H5N1 already stimulated to form, although it is not enough to show clinical symptoms [15]. Poultry that was positively detected can survive without dying and act as a reservoir for spreading AI through cloaca secretion/feces [15].

Table 2. Seroprevalence antibody titer of Avian Influenza H5N1 at Silir Poultry Market and Jumantono Farm

| Location                | Seroprevalence                      |
|-------------------------|-------------------------------------|
|                         | Positive (protective) | Negative (non-protective) | Total   |
| Silir poultry market    | 3 (4.28%)                | 67 (95.72%)                | 70 (100%) |
| Jumantono farm          | 34 (48.57%)              | 36 (51.43%)                | 70 (100%) |
| Total                   | 37 (26.43%)              | 103 (73.57%)               | 140 (100%) |

Environmental factors also influence the viral infection of AI subtypes H5N1 and H7N9. The spread of these subtypes related several factors, e.g., to the wild bird habitats, bird’s migrational behavior, and local climate [16]. An epidemiological study in AI cases infecting humans in China [17] showed that the viral infection was associated with the meteorological condition. Virus H5N1 is more adaptive to a temperature range of 2-22°C and an atmospheric pressure of 980-1025 kPa [17]. So far, there were no reports on the global climate change effects on the spread of AI viral infection in humans or poultry in Indonesia.

Even though AI H5N1 is highly pathogenic, it is susceptible to biosecurity control. Thus, biosecurity is a powerful tool to control and eradicate AI H5N1. Biosecurity can be defined as all actions as the first defense against the epidemic spread and performed to prevent all possible spread/contact with infected poultry to minimize the spread of disease [13]. WHO [14] explains that the main biosecurity components are isolation, traffic control, and sanitation. This study provides information on the chicken health status in two main poultry markets in Surakarta, Central Java. Hopefully, this data can be used as a guide to help farmers or merchants arrange prevention programs, proper disease treatment, and evaluation in future poultry farming.

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