SEROLOGICAL SURVEY ON *Avian influenza* SUBTYPE H5N1 VIRAL ANTIBODY IN HEALTHY BLOOD DONOR IN SURABAYA

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ABSTRAK

Penelitian ini bertujuan untuk melaksanakan survei serologis antibodi virus Avian influenza H5N1 pada darah donor sehat di Surabaya.Penelitian ini merupakan penelitian deskriptif untuk mengetahui titer antibodi virus Avian influenza H5N1 pada darah donor di Surabaya dan mengetahui serum yang mengandung antibodi dapat menetralisasi efek sitopatik dari virus Avian influenza H5N1. Sampel darah donor sebanyak 773 spesimen darah yang diambil dari Palang Merah Indonesia Surabaya dan diproses di laboratorium BSL-3 Avian influenza dengan metode Hambatan Haemaglutinasi (HI) dan Netralisasi. Hasil penelitian menunjukkan dari 773 darah donor didapatkan sebanyak 13 darah donor yang memiliki antibodi terhadap Avian influenza subtipe H5. Titer antibodi 1:10 untuk golongan darah A sebanyak 2 orang, golongan darah B sebanyak 1 orang, dan golongan darah O sebanyak 2 orang. Antibodi dengan titer 1:20 ditemukan pada golongan darah A sebanyak 4 orang, golongan darah B sebanyak 1 orang, golongan darah AB sebanyak 1 orang dan golongan darah O sebanyak 1 orang, sedangkan titer 1:40 hanya 1 orang dengan golongan darah O. Berdasarkan hasil pengujian dengan menggunakan uji netralisasi pada serum tidak ditemukan antibodi yang dapat menetralisasi efek sitopatik dari virus Avian influenza H5N1. Saran yang dapat diberikan berdasarkan hasil penelitian ini adalah diperlukan penelitian lanjutan untuk mendeteksi adanya virus Avian influenza dari subtipe lain dan dilakukan penelitian adanya hubungan antara virus Avian influenza pada darah donor dengan golongan darah.

ABSTRACT

The purpose of this study is to research serosurvey Avian influenza H5N1 antibody in healthy blood donor at Surabaya. This research is descriptive study to found prevalence and titre of antibody existence Avian influenza H5N1 antibody in blood donor at Surabaya and antibody in sera healthy blood donor can be neutralized cytopathic effect Avian influenza H5N1 virus. Total sample blood donor that inspected amount 773 specimen blood from Red Blood Cross Surabaya and processed at BSL-3 Avian influenza laboratory with Haemaglutination Inhibition (HI) and neutralization method. Result showed 13 blood donors from 773 blood donor have Avian influenza subtype H5 antibody. Antibody titre of subtype H5 1: 10, 2 people of blood type A, 1 people of blood type B and 2 people of blood type O. Antibody titre 1: 20 found 4 people of blood type A, 1 people of blood type B,1 people of blood type AB and 1 people of blood type O. antibody titre 1: 40 only 1 people with blood type O. This research did not found antibody in sera blood donor which can be neutralized cytopathic effect of Avian influenza virus subtype H5N1. Suggestions of this research will be needed detection antibody from other subtype of Avian influenza virus and do research for analyzed correlation between Avian influenza with blood group.

Keywords : *Avian influenza H5N1, blood donor, antibody titre*

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INTRODUCTION

Avian influenza, otherwise known as fowl plague was first discovered in Italy in 1878 as a disease that affects poultry (Werner 2006). *Avian influenza* is a zoonotic disease caused by type A influenza viruses of the family Orthomyxoviridae. *Avian influenza* virus subtype highly pathogenic H5N1 strain that can attack poultry (birds, ducks and chickens) throughout Southeast Asia and unexpectedly transmission from birds to mammals (cats, pigs and humans) (Rahardjo and Nidom 2004, Webster and Hulse 2004). Transmission from birds to cats proved by the discovery of *Avian influenza* H5N1 virus in cats in the street market area in the region of Semarang (Dwiyanto 2008). Yamaoka et.al (2008) has conducted surveillance of *Avian influenza* virus subtype H5N1 in poultry in Surabaya and Reviany et.al (2008) in poultry, cats and pigs in Indonesia. The results obtained indicate the presence of *Avian influenza* viruses that infect chickens and other poultry in Surabaya in Indonesia is even the A/Ck/Ind/114/2008 H5N1 subtype (H5N1). A/Ck/Ind/114/2008 virus (H5N1) has a similarity cluster with a virus that infects and causes fatalities in humans in Greater Jakarta.

Avian influenza virus subtype H5N1 is a virus that causes infections in humans and cause the degree of severity highly varied, ranging from infections that are asymptomatic until the disease is fatal and multisystemic (Suarez et al. 2004, WHO 2007). No fewer than 417 people have been reportedly infected with *Avian influenza* virus H5N1 and 254 of them died. In Indonesia, according to Health Ministry figures of people infected with bird flu virus as many as 141 people up to 8 April 2009 and 115 fatalities (WHO 2009). But until now there is still no scientific evidence indicating the existence of inter-human transmission (Saif and Espinoza 2006).

Manifestations of H5N1 *Avian influenza* that infects humans primarily in the respiratory tract through the air containing the virus or with the droplet infection of poultry. Transmission of infection can also be through saliva, nasal secretions, feces, blood and direct contact with body fluids contaminated by the virus *Avian influenza* (CDC 2006). Khakpour et.al (1969) *Avian influenza* viruses detected in the blood of an infected person with no symptoms (asymptomatic). Alamudi, et.al (2008) reported 136 people were merchants from avian poultry sellers in traditional markets and cutting Animal House (RPH) in Surabaya, three of them indicated the presence of antibodies against *Avian influenza* virus H5N1.

During this time the incidence of transmission of *Avian influenza* directly through the blood is not yet widely known (Norris 2003). This is due to a virus in the blood is rarely detected during the symptoms of *Avian influenza* infection, but viremia may occur symptomatic or asymptomatic influenza infection (WHO 2006). Chances are there are donors with asymptomatic viremia can potentially transmit the virus *Avian influenza* through blood or blood products. *Avian influenza* in the blood causes an increase in transmission of *Avian influenza* virus through blood transfusion (AABB 2006).

Detection of *Avian influenza* H5N1 virus antibodies in blood donors in Surabaya Indonesia Red Cross now has not been done, so that the required monitoring or surveys in the area or where it can be a transmission of *Avian influenza* virus subtype H5N1 in humans. Starting from this background, it is necessary to serological surveys to determine the presence of antibodies against *Avian influenza* virus H5N1 in healthy blood donors in Surabaya.

This study aims to prove there are antibodies against *Avian influenza* virus H5N1 in healthy blood donors in Surabaya, knowing the titer of antibodies against *Avian influenza* virus in the blood of healthy donors are positive, and prove that the serum of healthy donor blood contains antibodies that can neutralize the cytopathic effect of virus *Avian influenza* H5N1. Benefits of the research is expected to provide information about the results of serological survey of *Avian influenza* virus H5N1 antibodies in healthy blood donors in Surabaya, so can be used to inform the authorities and the results of serological surveys can be developed by other researchers to identify *Avian influenza* virus of subtype other.

MATERIALS AND METHODS

Test performed hemagglutination (HA) antigen titer microtechnique to know. Antigen used was a strain of *Avian influenza* A/Chicken/Indonesia/114/2008 (H5N1). At 1:64 titer obtained HA test. Barriers hemagglutination test was then performed (HI) Microtechnique, ie serology for the detection of specific antibodies *Avian influenza* virus subtype H5. HI test results were 13 blood donors who have antibodies H5 subtype. HI test with a titer of 1:10 as many as 5 people. HI titer test with titers of 1:20 and 1:40 sebanyak7 people only 1 person.

Tues inoculation MDCK (Madin-Darby Canine Kidney) in Tissue Culture Plate

Cells in flasks were washed with PBS. Tues back in the wash with PBS and Trypsin-EDTA and shaken flasks so that the cells flushed. Add a few drops of Trypsin-EDTA and incubation at a temperature of 35-36°C 5% CO2 for 10 minutes, then cells were observed in the microscope. Add Trypsin-EDTA. Cell suspension was removed and centrifuged. Supernatant was added to growth medium. Enter the cell suspension of each hole 48 well plate. Furthermore, growth medium was added and the incubation at 37°C 5% CO2 until confluent cell monolayer.

Test TCID50 (Tissue Culture Infectious Dose 50)

After a confluent cell monolayer in the well plate, then do the washing with PBS 3 times. Enter the PBS into the microtube 10-1-10-100 7. The virus was added into the microtube 10-1 and performed serial dilutions. After that insert a virus dilution of 100 mL each hole corresponding dilution in 48 well plate and the last hole filled with PBS as negative control. Incubation at 37°C 5% CO2 for 60 minutes, every 20 minutes on a rocking plate. Add the mixture of maintenance medium with TPCK trypsin per hole. Incubation at 37°C 5% CO2 and check for CPE (Cytopathic Effect) every day. After 4 days the medium of all the holes move and perform

fixation of cells by inserting a 10% formalin and incubation at room temperature for 30-60 minutes. Furthermore, formalin waste from all the holes and washed with water. Perform staining cells with crystal violet for 5 minutes and washed with water. TCID50 using Reed and Muench formula results obtained for 63.246 x 104/ml which is the dilution of virus that can kill 50% of MDCK cells.

Virus Neutralization Test
(Virus Neutralization Assay)

Cells in flasks washed with PBS and Trypsin-EDTA and shaken flasks until the cells flushed. Add Trypsin-EDTA and incubation at a temperature of 35-36°C 5% CO2 until the cells detached and were observed in the microscope. Tues Trypsin-EDTA was added in 5 ml of cell suspension and centrifuged. Supernatant was added to growth medium, until the cell does not clot. Enter the cell suspension per 96 well plate. Furthermore plate filled with growth medium. If the cell clumping then shaken until the cells separate plate. Incubation at a temperature of 35-36°C 5% CO2 for 18-24 hours. Serum to be examined in the inactivation at a temperature of 56°C. Prepare microtiter plate and insert the virus growth medium (VGM) to all the hole plate. Add VGM back to the line A (hole A1-A11). Insert-1 serum to each hole in the row A (A1-A4), then serum-2 on line A (A5-A8) and do the serial dilution.

Conducted 100 TCID50 virus dilution to 50 mL with virus growth medium (VGM). Enter the virus dilution to 60 mL every hole except all holes H as a control cell line filled VGM. Titration was then performed back. Insert into the hole BH VGM, then add 438 mL of dilutions of the virus into the hole and do a serial dilution. This result is ½ log 10 serial dilutions of virus. VGM enter into any hole microtiter plate at A9-A12. Then move back 60 mL Titration of virus from A9-A12, B9-B12 and so on. Shake and incubation temperature of 35-36°C 5% CO2 for 2 hours. 96 well plates were washed with PBS and Back Titration Virus inoculation with a mixture of serum and virus transfer from plate to 96 well microtiter plate each and every move the hole using a different pipette tips. Incubation temperature of 35-36°C 5% CO2 for 2 hours. Tues washed with maintenance medium and discarded. The entire hole is filled with maintenance medium and incubation temperature of 35-36°C 5% CO2 selama3-4 days. After that fixation with formalin 10% and incubation at room temperature for 30-60 minutes, and then washed with water. Perform staining with crystal violet for 5 minutes. The results obtained throughout the sample obtained neutralization test result is negative and MDCK cells had visible CPE (Cytopathic Effect)

RESULTS

The results of descriptive data of this study to determine the prevalence and titers of antibodies *Avian influenza* virus H5N1 in healthy blood donors in Surabaya. Donor blood sampling was conducted from October to November 2008 at the PMI Surabaya.

Table 1. Blood Sample Data Based Blood Donor Classified

Blood Groups	Total sample (blood donors)	Percentage (%)
A	136	17.59 %
B	240	31.05 %
AB	49	6.34 %
O	348	45.02 %
Total	773	100%

Results Barriers hemagglutination (HI) Microtechnique

Barriers hemagglutination test (HI) is the serological testing to detect the presence of antibodies *Avian influenza* virus subtype H5. Microtechnique HI assay results from 773 blood donors obtained as many as 13 blood donors who had antibodies to H5 subtype and 760 blood donors who do not have antibodies against subtype H5 HI test.

Table 2. The Blood Donor Data Positive Test Against Barriers hemagglutination (HI)

No	Sample codes	Blood group	HI test (Titer)
1	H0810558	A	1:10
2	H0810559	A	1:10
3	H0810527	A	1:20
4	H0810561	A	1:20
5	H0810760	A	1:20
6	H0810192	A	1:20
7	H0810555	B	1:10
8	H0810562	B	1:20
9	H0810193	AB	1:20
10	H0810316	O	1:10
11	H0810554	O	1:10
12	H0810526	O	1:20
13	H0810289	O	1:40

Test results TCID50
(Tissue Culture Infectious Dose 50)

In this study tested the TCID50 (Tissue Culture Infectious Dose 50) on the *Avian influenza* virus strain A/Chicken/Indonesia/114/2008 (H5N1). TCID50 calculation results will then be used for the calculation of back Titration virus in neutralization tests. TCID50 calculation using Reed and Muench formula. The results

obtained TCID50 of H5N1 A/Chicken/Indonesia/ 114/2008 63.246 x 104/ml is the result of dilution of virus that can kill at least 50% of MDCK cells. TCID50 assay results in MDCK cells appear experiencing CPE (Cytopathic Effect) due to *Avian influenza* virus H5N1. After incubation, indicating that almost all the cells shrink and are not visible cell nucleus and unable to absorb the crystal violet staining (arrow) as shown below:

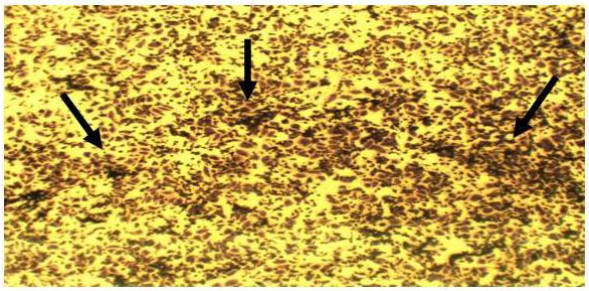

Figure 1. Test Results TCID50 In A/H5N1 Chicken/Indonesia/114/2008

Neutralization Test Results

Positive results in the neutralization test indicated the presence of specific antibodies capable of neutralizing the virus *Avian influenza* in MDCK cells (Madin-Darby Canine Kidney Cells). In 13 samples of blood donors from the HI test followed by a neutralization test (Table 3).

Table 3. Results Blood Donor Against *Avian influenza* Virus Subtype H5N1 With Neutralization Test

No	Sample Codes	Blood Groups	HI (titer) Test	Neutralization Test (titer)
1	H0810558	A	1:10	Negative
2	H0810559	A	1:10	Negative
3	H0810527	A	1:20	Negative
4	H0810760	A	1:20	Negative
5	H0810561	A	1:20	Negative
6	H0810192	A	1:20	Negative
7	H0810555	B	1:10	Negative
8	H0810562	B	1:20	Negative
9	H0810193	AB	1:20	Negative
10	H0810316	O	1:10	Negative
11	H0810554	O	1:10	Negative
12	H0810526	O	1:20	Negative
13	H0810289	O	1:40	Negative

Analysis of the neutralization test against 13 blood donors, acquired the entire sample was negative. In the neutralization test negative MDCK cells have shown CPE (Cytopathic Effect) due to *Avian influenza* virus H5N1. After the incubation, indicating that almost all the cells shrink and are not visible cell nucleus and unable to absorb the crystal violet staining.

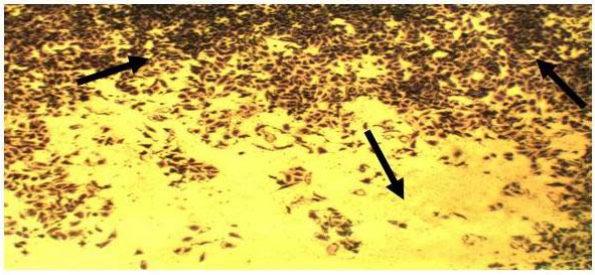

Figure 2. Neutralization Test Results Negative In cells MDCK

MDCK cells undergo apoptosis because in the serum of blood donors, so there are no specific antibodies can not neutralize the *Avian influenza* virus H5N1. Tues MDCK who experience CPE (Cytopathic Effect) is seen in the presence of cells that contract, not seen most of the cell nucleus and apart from that cell lysis could not absorb crystal violet staining (Figure 2). In this research can be proved that the HI test, there were 13 blood donors who have a tendency to low titers of antibodies against *Avian influenza* virus subtype H5. In the neutralization test of 13 blood donors there are no specific antibodies against *Avian influenza* virus H5N1. In the neutralization test results visible MDCK cells had CPE (Cytopathic Effect), this is because the antibodies present in serum of blood donors are not able to neutralize the *Avian influenza* virus H5N1.

DISCUSSION

The test study was conducted in BSL-3 Laboratory of *Avian influenza* Airlangga University. Based on the results of resistance testing hemagglutination (HI) is known to the overall prevalence of 0.776% of blood donors with blood group A antibodies against *Avian influenza* subtype H5 (6 blood donor blood group A), 0.258% of blood donors blood type B have antibodies against *Avian influenza* H5 subtype (2 blood donor blood group B), 0.129% blood type AB blood donors have antibodies to *Avian influenza* subtype H5 (a blood donor blood group AB), and 0.517% of blood donors blood type O have antibodies against *Avian influenza* subtype H5 (4 Blood Type O blood donors).

The results of measurements of antibody titers in 13 samples of blood donors by using the HI test between 1:10, 1:20 and 1:10 to 1:40 is the blood group A as much as 2 people, blood group B as much as a person, and O blood type as much as 2 people. Antibodies with titers of 1:20, the blood group A as much as 4 people, blood type B by 1 person, blood type AB as much as a

person and as a blood type O people, whereas titers of 1:40 only a person with blood type O.

This suggests the presence of antibodies against *Avian influenza* virus in blood donors in Surabaya PMI which has been the detection of *Avian influenza* virus in blood donors has not been done because of possible donors potentially transmit *Avian influenza* virus through blood or blood products can occur. The existence of the relationship between *Avian influenza* virus in blood donors with blood group until now has not been done the research. In this study of blood donors obtained from the prospective donor/volunteer is a population of low-risk communities against *Avian influenza* H5N1 virus infection, it is different from the research conducted by Alamudi, et. al (2008) whose population is derived from poultry traders who may have a high risk of infection by *Avian influenza* virus H5N1. Presence of *Avian influenza* virus in the blood donor corresponds to the statement put forward by Khakpour et.al (1969) that *Avian influenza* viruses have been found in the blood of people with no symptoms (asymptomatic).

Antibodies are the main components of the specific immune response to protect against influenza virus. *Avian influenza* virus glycoproteins form of hemagglutinin (H) is an attachment to the host cell surface receptors and these proteins can cause an immune response in the form of the initial target in the formation of antibodies (HL Yen and Peiris 2009). Hemagglutinin (H) in addition to determining the infectivity of influenza viruses also can spur the onset of immune responses in the host because it is antigenic and immunogenic. Antibodies formed play an important role in protecting from virus infection and induces antibodies to neutralize the virus. These antibodies will inhibit the virus before getting into the cells early in infection and viral exit from infected cells by binding to the virus then the virus uncoating. (Treanor 2004, Hunt 2006, Abbas 2007).

Based on the structure of the amino acids that make up the cleavage site (cleavage site) gene hemagglutinin (H) can be distinguished on the *Avian influenza* virus and the virulent virus avirulen. *Avian influenza* virus H5N1 is a virulent virus that has several amino acids that are categorized as Highly Pathogenic *Avian influenza* (HPAI). The existence of poly amino acids in the region of cleavage site is the target of proteases and plasmin that can cause the virus to spread systemically throughout the body and increase the virulence (Horimoto and Kawaoka 2001).

Tests using the Hemagglutination Inhibition (HI) and neutralization is the serological methods used in detecting the presence of *Avian influenza* viruses. Agglutination reaction barriers (HI) can assist in identifying the laboratory diagnosis of virus neutralization test whereas a 'gold standard' for detection of *Avian influenza* virus H5N1 antibodies (De Jong et al. 2006, Uyeki 2008). Both serological methods is important in conducting epidemiological studies and surveillance of *Avian influenza* antibodies (WHO 2005, Ali et al. 2007). Seroepidemiology studies used for the purposes of determining the transmission and risk factors associated with *Avian influenza* H5N1 virus infection in humans (Rowe 1999).

The discovery of donor blood that contains antibodies by HI test showed that the donor was never exposed to the *Avian influenza*, but it is unknown donor has not entered the acute phase after a primary infection. This was likely caused when the blood specimen is taken, the donor in the state of antibody has not been formed so that a low antibody titer. According to FAO (2006) WHO standards for investigation and monitoring of *Avian influenza* virus incubation period is 7 days after infection. This assumption is reinforced by research conducted Rowe et al. (1999) using serum of less than 7 days after initial infection and the acquired antibody titers low at less than 20. In the HI test to detect antibodies of H5 subtype *Avian influenza* virus, the serum is more than 14 days because of the possibility of getting a positive result is greater than the detection of antibodies in the serum after primary infection of less than 7 days.

In this study, the serum of blood donors found no specific antibodies against *Avian influenza* H5N1 virus by using a neutralization test. This is possible because of donors who donate blood in a state of less than 7 days after initial infection. Conversely, if the donor's blood serum was detected more than 14 days after infection, or more, the possibility of a positive antibody obtained. This statement is evidenced by the results of research conducted by the neutralization test Katz et al. (1999) who obtain a positive antibody titer for 80-2560 from serum obtained from adult patients who had more than 14 days had clinical symptoms and positive antibody titers more than 640 of serum from children and adults who take more than 20 days after the clinical symptoms.

According to De Jong et al. (2006) no detection of antibodies in serum by using the HI test can be attributed to several factors, among others, in some *Avian influenza* viruses the immunogenity low that the HI test can detect, low sensitivity in detecting low titers of antibodies with the antibodies as well as having low avidity. This confirms that by using the neutralization test is the recommended serological methods to detect specific antibodies *Avian influenza* H5N1 virus in the

serum of adults because it is more sensitive and specific than the HI test. Another advantage of the neutralization test is more sensitive in the detection of influenza virus types A and B in humans and is able to detect the H5 subtype antibodies in the serum of children in the acute phase serum taken more than 7 days after initial infection or in the convalescent phase serum obtained after more than 14 days had clinical symptoms (Rowe et al. 1999, De Jong et al. 2006). According to Rowe et al. (1999) on the neutralization test is less specific in detecting antibodies in serum in people older than 60 years, this is probably caused because of age imunocompromised by someone who has advanced to allow a decline in immune system. Research conducted Katz et al. (1999) of women who suffer from SLE (Systemic Lupus Erythematosus) was also not detected antibodies to the neutralization test.

Blood donors who have antibodies indicate the presence of *Avian influenza* virus transmission to humans from infected birds that can occur through direct contact or from contaminated environmental factors, but if there is no contact with infected birds then there is the possibility of direct physical contact with patients who can cause the transmission of *Avian influenza* H5N1 virus among humans (Katz et al. 1999, Harner and Werner 2006, Kamps and Reyes Teran 2006, WHO 2008).

Detection of blood donors who have a tendency to low titers of antibodies against *Avian influenza* virus subtypes H5 and do not have specific antibodies against *Avian influenza* virus H5N1, this is possible due to several factors including donor blood serum obtained from donors who have experienced symptoms of infection is less than 7 days. Limited data is also a limiting factor donors can therefore not be further surveyed donors who have antibodies to *Avian influenza* virus.

CONCLUSION

Contained antibodies against *Avian influenza* virus subtype H5 in healthy blood donors in Surabaya and was not found specific antibodies that can neutralize the cytopathic effects of *Avian influenza* virus H5N1. Further research is needed to detect the presence of *Avian influenza* viruses of other subtypes and required other methods such as ELISA or PCR to detect *Avian influenza* virus H5N1. In addition it is necessary to study further the relationship between *Avian influenza* virus in blood donors with blood group. Similarly, cooperation with the Indonesian Red Cross (PMI) is needed for surveillance and monitoring of *Avian influenza* H5N1 virus in blood donors.

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