THESIS

CROSS REACTIVITY BETWEEN H5 CLADE 2.1.3 SERUM AND H5 CLADE 2.3.2 VIRUS BY USING SERUM NETRALIZATION TEST

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DECLARATION

Hereby, I declare that in this thesis entitled:

CROSS REACTIVITY BETWEEN H5 CLADE 2.1.3 SERUM AND H5 CLADE 2.3.2 VIRUS BY USING SERUM NETRALIZATION TEST

There is no work ever published to obtain a college degree in a certain college and to my knowledge there is also no work or opinion ever written or published by others, except those in writing referred to in this paper and mentioned in the references.

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CROSS REACTIVITY BETWEEN H5 CLADE 2.1.3 SERUM AND H5 CLADE 2.3.2 VIRUS BY USING SERUM NETRALIZATION TEST

Muhammad Khaliim Jati Kusala

ABSTRACT

The aim of this research was to know the cross-reactions between avian influenza virus clade 2.1.3 and 2.3.2 to 2.1.3 clade avian influenza serum by using serum neutralization test also to know the effectivity avian influenza clade 2.1.3 vaccine against 2.1.3 and 2.3.2 virus in vitro. This research was held at Avian Influenza Research Center (AIRC) with the laboratory Biosafety Level-2 (BSL-2) Universitas Airlangga. This research was held from June to July 2014. Serum of H5N1 clade 2.1.3 that be used had the titre of 2^7 by Haemaglutination Inhibition (HI) assay. The method of this research is using H5 clade 2.1.3 serum stock AIRC Universitas Airlangga that be tested against H5 clade 2.1.3 (A/Ck/Ind/114/08) and 2.3.2 (A/Dk/Ind/443/12) virus by using Serum Neutralization Test assay. The TCID50 of the viruses are 1.78 x 108 TCID50/ml of H5 clade 2.1.3 and 5.62 x 107 TCID50/ml of H5 clade 2.3.2. Serum was tested with both viruses by using serum neutralization test (SNT) assay in the MDCK cell culture. The data was analyzed by Reed and Muench Formula. The result of SNT showed that the serum were able to neutralize and protect at least 50% of cell culture at the 1:160 dilution to H5 clade 2.1.3 virus. While serum tested with H5 clade 2.3.2 virus on SNT, it was showed that there were cross reaction, the serum was able to neutralize the virus and protect at least 50% of cell culture at the 1:1258 dilutions to H5 clade 2.3.2 virus.

Key words: Avian influenza, H5N1 clade 2.3.2 Vaccine, Serum Neutralization Test (SNT), Cross Rectivity.

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ABBREVATIONS AND SYMBOL MEANING

AI	= Avian Influenza
BSA	= Bovine Serum Albumine
CO2	= Carbondioxyde
CPE	= Cytophatic Effect
DNA	= Deoxyribonucleic Acid
D-MEM	= Dulbecco's Modified Eagle Medium
DW	= Distillated Water
EDTA	= Ethylene Diamine Tetra Acetic
FBS	= Fetal Bovine Serum
H	= Hemagglutinin
HA	= Hemagglutination
HAU	= Hemagglutination Unit
HI	= Hemagglutination Inhibition
HPAI	= Highly Pathogenic Avian Influenza
LPAI	= Low Pathogenic Avian Influenza
MP	= Matrix Protein
MDCK Cell	= Madin-Darby Canine Kidney Cell
MEM	= Minimum Esential Medium
ml	= Mililiter
MM	= Maintenance Medium
NA	= Neuraminidase
NP	= Nucleoprotein
NS	= Non Structural
OIE	= Office Internasional des Epizooties
PB	= Polymerase Basic
pH	= Power of Hydrogen
PBS	= Phosphat Buffer Saline

RBC = Red Blood Cell
RDE = Receptor Destroying Enzyme
RNA = Ribonucleid Acid
RNP = Ribonucleoprotein
rpm = Rotation per Minute
SNT = Serum Netralization Test
TCID50 = Tissue Culture Infective Dose 50
WHO = World Health Organization
µl = Microliter

CHAPTER 1

INTRODUCTION

CHAPTER 1 INTRODUCTION

1.1 Background

Avian Influenza is belonged to the H5N1 subtype of Influenza A virus which can infect humans and many other animal species. Although the outbreak of Avian Influenza in Indonesia had been abated, but awareness of the case are still needed, because its emergence can occur repeatedly. In fact, in some countries, in 1-2 decades has occurred at least three (3) times outbreak of the Avian Influenza virus, which is around 1997, 2003-2004, and the latter toward the end of 2012. Meanwhile in Indonesia Avian Influenza outbreak began in 2003. Though the virus is usually transmitted between birds and chickens, but in fact, the virus has infected ducks and other types of animals such as pigs, horses, and cats. Even more frightening is the zoonotic of avian influenza viruses that capable to infecting humans and can even lead to death (Legowo, 2013).

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. There are three influenza genera – A, B and C, but only influenza A viruses are known to infect birds (OIE, 2012).

Influenza A viruses are the only Orthomyxoviruses known to naturally affect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses, aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for

chickens and turkeys. Influenza A viruses have antigenically related nucleocapsid and matrix proteins, but this virus are classified into subtypes on the basis of their haemaglutinin (H) and neuraminidase (N) antigens (World Health Organization Expert Committee, 1980).

Because the characteristics of the influenza virus constantly evolve and change unpredictably, it is very important to control the virus. The influenza A virus is an RNA virus with eight gene segments. At present, there are 18 H subtypes (H1-H18) and 10 N subtypes (N1-N10) has been known (Tong et al, 2013; Swayne and Halvorson, 2008). Influenza A viruses are highly lethal can cause acute clinical disease in chickens, turkeys and other avian species of economic interest has been associated only with subtypes H5 and H7. Actually, most viruses of subtypes H5 and H7 has a low virulence for poultry. However, the risk of influenza A virus subtypes H5 or H7 that has low virulence can be turned into high virulence due to genetic mutation (Vivi *et al*, 2011).

Highly pathogenic avian influenza A (H5N1) virus has circulated in poultry in Indonesia since 2003. These viruses became enzootic and evolved into second-, third-, and fourth-order HA clades, leading to the recent dominance, that is clade 2.1.3 viruses. Outbreaks in poultry typically caused high mortality rates among gallinaceous birds, especially layer, broiler, and native chickens. The virus seemed less pathogenic in aquatic birds (Dharmayanti et al, 2012).

In Indonesia, there are three sub-clade of Avian Influenza virus, that is a sub-clade 2.1.1, 2.1.2, and 2.1.3. If there are gene mutations, the possibility of its sub-

clade is 2.1.1.1 or 2.1.2.1 or become 2.1.3.1. So, if discovered sub-clade 2.3.2 means that the process is too fast and the possibility is small and the most possible is a sub-clade 2.3.2 is come from outside Indonesia (Nidom, 2012)

Avian Influenza H5N1 virus subgroup (subclade) 2.3.2 and 2.1.3 showed high pathogenicity. Avian Influenza subclade 2.3.2 which attack the ducks in the end of 2012 have the same pathogenecity with Avian Influenza subclade 2.1.3 that killed thousands of birds and 180 people in Indonesia since a few years ago (Veterinary Research Institute, 2013).

Influenza A virus vaccines, including vaccines Avian influenza virus is a way that can be used to prevent and control influenza A virus infection. The influenza vaccine can induce neutralizing antibody response to the vaccine strains and isolates that are closely linked or identical, but rarely extends to different strains within a subtype or other subtypes. Therefore, vaccines for influenza A, especially for the human, at this time is always prepared every year based on the estimation of the World Health Organization (WHO) to the influenza virus strains that are considered most likely can be circulating in the next seasonal outbreaks (Zhou, 2012)

But to choose the right vaccine strain, has many challenges and sometimes lead to suboptimal protection. Moreover, predicting the next pandemic virus, including when and where will arise, is currently difficult. Thus, developing a vaccine "universal" that could lead to an antibody response capable of neutralizing influenza A virus strains which vary will eliminate a lot of the uncertainty associated with the

selection of certain viral strains and can inhibit the emergence of a pandemic virus (Zhou et al, 2012).

Likewise, with the development of sublcade AI virus in Indonesia, while the corresponding AI vaccine has been no study, especially vaccines derived from AI viruses clade 2.1.3 can neutralize the virus subclade 2.3.2 subclde. Based on these conditions, the study was conducted for the analysis of antibody derived from AI virus vaccine subclade 2.1.3 against H5N1 Avian Influenza Virus subclade 2.1.3 and 2.3.2. The analysis will be carried out by cross-reaction test using the serum neutralization test (SNT). It is hoped that this research can be used to assist in determining the appropriate vaccination strategy against Avian Influenza Virus in the future.

1.2 The Problems Statements

Is there cross-reactions between avian influenza virus clade 2.1.3 and 2.3.2 to 2.1.3 clade avian influenza serum by using serum neutralization test?

1.3 Theoritical Base

Avian influenza (AI) is caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenza virus A. Each segment of Influenza type A virus consists of a protein Polymerase component 2 (PB2), polymerase component 1 (PB1) and Polymerase component (PA) which encodes Polymerase, Haemaglutinin (HA), nucleocapsid (NP), Neuraminidase (NA), matrix

protein 1 (M1), Matrix Protein 2 (M2), Non- Structural Protein 1 (NS1) and Non-Structural Protein 2 (N2). These proteins have their respective roles to life Influenza virus type A. HA protein plays a role in the process direct interaction with the receptor is in cell surface (attachment). While the NA protein role in the release of virus from cells (budding) (OIE, 2012).

Avian Influenza virus has a characteristic easily to mutation so that it can make the situation become more pathogenic virus or less pathogenic (Dharmayanti, *et al.* 2008). Genetic mutation of avian influenza virus often occurs in accordance with the conditions and environment. Mutations of this gene is not only to defend themselves but also can improve the properties of pathogenicity (Seo, *et al.* 2002). Prevention of avian influenza outbreaks, especially to prevent the transmission to human can be done effectively by increasing the "biosecurity", the second method is through vaccination of domestic poultry. This method is considered as the most likely way that applied at this time, because it is effective and relatively inexpensive (Niloperbowo, 2013).

Serological test is a method used to know the antibody titer in the chicken's body. One of the serological Tests to determine the ability of an antibody is serum neutralization test (SNT). The serum neutralization test is highly sensitive and specific assay for detecting virus-spesific neutralizing antibodies to influenza viruses in human and animal sera (WHO, 2011). The working principle is the reaction of virus neutralization assay with serum containing antibodies then inoculated to the host system such as cell culture or Chicken Eggs Embryonated. Visualization of virus

neutralization activity can be detected in the presence of Cythopathic effect on cells (WHO, 2002; Indrasari, 2013).

1.4 The Aim of The Research

To know the cross-reactions between avian influenza virus clade 2.1.3 and 2.3.2 to 2.1.3 clade avian influenza serum by using serum neutralization test.

1.5 The Outcomes of the Research

1. To give information about cross reactivity between avian influenza serum clade 2.1.3 to avian influenza virus clade 2.1.3 and 2.3.2 so this research can be used to assist in determining the proper vaccination against Avian Influenza Virus in the future.
2. Can be used to know how to test the results of a vaccine as a substitute challenge test.

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2 LITERATURE REVIEW

2.1 Overview of Influenza Virus

The influenza virus is an RNA virus that include in the family Orthomyxoviridae. Viral nucleic acid is single-stranded, composed of eight gene segments that encode approximately 11 proteins. Influenza viruses have a sheath / hoop consisting of a complex of proteins and carbohydrates. This virus has protrusions (spikes) that are used to attach to specific receptors on cells when infecting the host cells. There are 2 types of spikes containing hemagglutinin (HA) and neuraminidase (NA), which is located at the outermost of the virion. Influenza viruses have 4 types of antigens consisting of (i) the nucleocapsid protein (NP) (ii). Hemagglutinin (HA), (iii). Neuraminidase (NA), and matrix protein (MP). Based on the types of antigens NP and MP, influenza viruses are classified in the influenza virus A, B, and C. (Horimoto, Kawaoka. 2001; Radji, 2006).

Influenza A viruses are very important in the life society because it is very pathogen for humans and animals, which cause substantial morbidity and high mortality in the worldwide. Influenza A viruses can cause pandemics because they easily to mutate, either antigenic drift or antigenic shift so as to form new variants are more pathogenic. There are 18 subtypes of HA and 10 NA subtypes. The seroprevalence of various epidemiological research suggests that some subtypes of influenza A viruses have caused outbreaks for examples like H7N7 pandemic (1977),

H3N2 (1968), H2N2 (1957), H1N1 (1918), H3N8 (1900), and H2N2 (1889) (Yuen and Wong, 2005; Radji, 2006; Tong et al, 2013).

Influenza B virus is a type of virus that only infect humans, whereas influenza C virus, are rare, although it can cause infections in humans and animals. Type B and C influenza viruses rarely cause outbreaks or pandemic (Horimoto, Kawaoka. 2001; Radji, 2006).

2.2 Infection process and Replication of Influenza A Virus

Type A influenza virus is an RNA virus (ribonucleic acid) single stranded of negative sense (-ssRNA). What is meant by negative-sense is, the viral genetic material (RNA) is a partner / complement of the mRNA strand. In contrast to the mRNA that can be used to encode a protein,-ssRNA may encode proteins after converted to + ssRNA (mRNA) (Niloperbowo, 2013).

Influenza A virus infection begins with the formation of a bond between the virus with the host cell (avian cells or human cells). This bond is facilitated by a lot of hemagglutinin that available in the surface of influenza A virus with many distinct terminal sialic acid receptors on the surface of cells in the respiratory tract . The bond between the Hemagglutinin with distinct terminal sialic acid receptors that cause the virus particle attaches to the host cell. Furthermore, the host cell perform endocytosis and then the virus will enter the cell in the form of the endosome (virus particles that covered by the host cell membrane) . As a part of the defense system, the host cell will destroys the virus inside the endosome by decreasing the acidity of the

endosome. However, when the pH of the endosome drops to 6.0, hemagglutinin on the surface of the virus will be unstable, partially decompose and release the "fusion peptide" that attaches firmly on the endosome membrane. This "Fusion peptide" then will bring the endosome membrane close with the membrane of the virus which then resulted in the two membranes to fuse. The fusion between the two membranes make the entire contents of the virus enter into the host cell cytoplasm (Niloperbowo, 2013).

After the material virus enters into the cell cytoplasm, then the virus begin the replication process, which begins with the synthesis of + ssRNA (mRNA) by using the - ssRNA which is the material genetic of influenza virus type A. This process is facilitated by the "RNA replicase" which is one of the contents from virus particles . After mRNA is formed, then by using the host cell translation system, the resulting mRNA is used to synthesize a variety of proteins needed to form a new virus . At the same time using the mRNA produced, performed well - ssRNA synthesis using "RNA replicase". When - ssRNA and proteins needed to form virus particles have been formed, the virus particles begin to form and ready to get out of the cell to infect other cells or animals. The release of newly formed virus progeny of the cells is facilitated by Neuraminidases (N) found on the surface of virus particles (Niloperbowo, 2013).

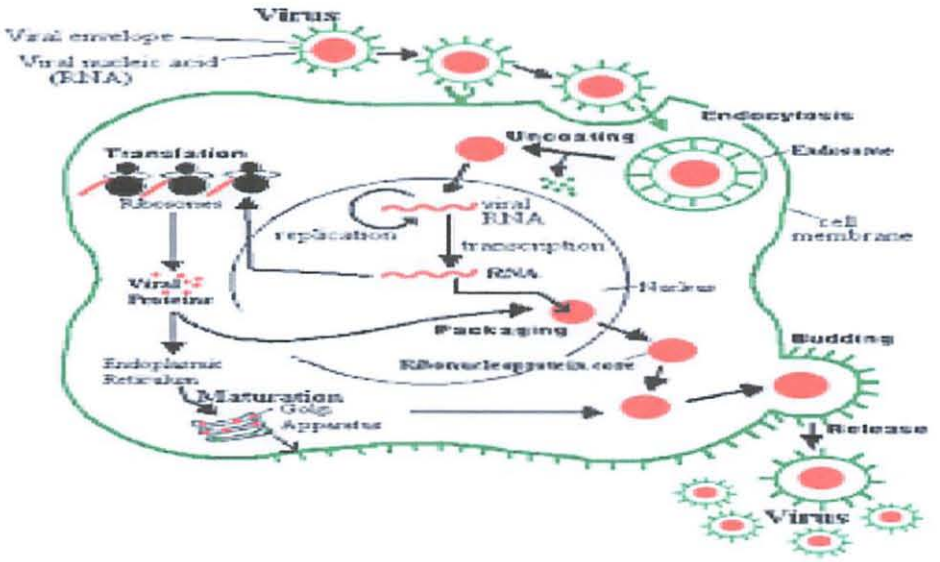

Figure 2.1. Infection and Replication Process of The Influenza A Virus
Source : Niloperbowo (2013)

If Neuramidase on the surface of the virus particle is compatible with the infected host cell, the virus can get out of the cells and then infect other cells or animals. However, if the virus Neuraminidase type is not compatible with the infected cell, the viral particles will remain in the cell. Scheme infection and replication of influenza virus type A can be seen in Figure 2.1 (Niloperbowo, 2013).

Unlike the DNA replication process that has a system that can ensure the correction of the error from the replication process, RNA replication process does not have this system, so that replication errors are not corrected. This is the cause of high mutations in RNA viruses. This mutation is very important to note, because the changes of the nucleotide sequence from the virus can lead to changes the character of the virus (Niloperbowo, 2013)

2.3 Avian Influenza Virus

Avian Influenza is a disease that caused by type A influenza viruses, which belong to the family Orthomyxoviridae. Influenza type A virus is a single stranded RNA virus that has enveloped with eight segments of negative polarity and round or filaments with a diameter 50-120 nm x 200-300 nm. Influenza type A viruses are found in birds, humans, pigs, horses and occasionally on mammals such as mink and whales (Dharmayanti and Hewajuli, 2008).

Avian influenza (AI) viruses are categorized into subtypes and pathotypes. This subtype differences based on serological types of two glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Eighteen different haemagglutinin antigens (H1 to H18) and ten neuraminidases of different antigens (N1 to N10) are now known and all have been isolated in wild birds (Alexander, 2007; Swane and Pantin - Jackwood, 2008; Tong *et al,* 2013).

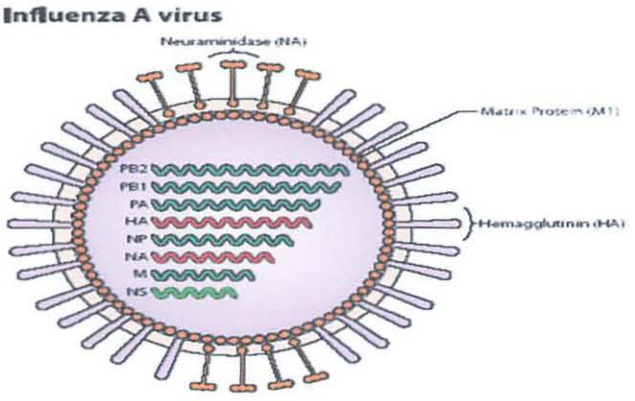

Figure 2.2 : Gene Structure of the Avian Influenza Virus
Source : Wellenberg (2006)

Based on the results of studies in genomics, recognized several subtypes of avian influenza, however, over the last 6 years only subtypes H5, H7 and H9 are known to spread from birds to humans (Liu et al, 2005).

Originally Avian Influenza virus is host specific , meaning that only a certain subtype of virus specific to a certain hospes. Host specific receptor is determined by the different structures between the hospes. Receptor sialic acid alpha 2,3-galaktose found in birds while sialic acid alpha 2,6-galactose found in humans. However, lately frequent reports of human cases of avian influenza caused by subtype H5N1. This happens because the avian influenza virus is able to mutate in two ways: antigenic drift and antigenic shift . Antigenic drift occurs due to minor changes in the antigens structure on the surface antigens HA or NA . The pattern of mutation mechanism through antigenic drift is only cause the addition or subtraction of nucleotide sequences of antigen HA, NA , or both without producing new virus subtype . Whereas antigenic shift occurs due to changes in the structure of the antigen that are dominant in the HA or NA surface antigens through the activity of two kinds of subtypes of avian influenza virus that is able to produce new viral subtype as a result of genetic recombination (Harder and Werner, 2006).

Avian Influenza virus has a characteristic easily to mutation so that it can make the situation become more pathogenic virus or less pathogenic. Avian Influenza Virus by pathogenicity can be divided into two that is Highly Pathogenic Avian Influenza (HPAI) and Low Pathogenic Avian Influenza (LPAI). HPAI characterized by nearly 100% mortality in birds, especially domestic poultry and race with or

without clinical symptoms before death. In Asia, Avian Influenza viruses subtype H5N1 including strains of HPAI viruses. Waterfowl and wild birds are the natural reservoir of HPAI, without showing clinical symptoms, so both these birds is one of its medium that can spread the virus HPAI strains and becoming increasingly widespread (Dharmayanti and Hewajuli, 2008).

While LPAI form indicated by milder clinical symptoms, including respiratory disorders, depression and a drop in egg production. Nevertheless, the LPAI virus strains can mutate into HPAI strains. The mutation process possibility from the wild birds transmitted to domestic poultry. Then the virus strains circulating for several months in domestic poultry. The results showed that the strain of LPAI virus mutated antigenic drift during circulating in poultry body. Based on this reason, the World Organization for Animal Health (OIE) is now define HPAI and LPAI naming system to only Avian Influenza. Avian influenza is included in the A list in the OIE, because Avian Influenza is a disease that is very dangerous to the animals and humans health (EID, 2006).

Avian Influenza virus remains infective in feces for 30-35 days at a temperature of 4°C and for 7 days at a temperature of 20°C. This suggests that the avian influenza virus can survive in the environment within a certain period of time and temperature. The properties enable the spread of avian influenza virus in nature. Transmission of Avian Influenza virus can occur through direct contact with a sick chicken to sensitive chicken. Infected birds excrete the virus from the respiratory tract, conjunctiva and feces. Transmission can also occur indirectly, for example

through contaminated air or dust material that contains a virus Avian Influenza (aerosol), food or beverages, tools or farm equipment, cages, clothing, vehicles, crates of eggs, egg tray, birds, mammals, and insects that contain Avian Influenza virus (Tabbu, 2000; Dharmayanti and Hewajuli, 2008).

2.4 Pathogenesis of Avian Influenza Virus

Genetic mutation of avian influenza virus often occurs in accordance with the conditions and environment. Mutations of this gene is not only to defend themselves but also can improve the properties of pathogenicity. A study of H5N1 viruses isolated from patients infected in 1997, showed that the genetic mutation at position 627 of the PB2 gene that encodes the protein expression of basic polymesase (Glu627Lys) has produced highly cleavable hemagglutinin glycoprotein that is a virulence factor that can increase the activity of the H5N1 virus replication in cells host (Hatta M, et. al. 2001). Besides that, the absence of substitution on nonstructural proteins (Asp92Glu), causing H5N1 resistant to interferon and tumor necrosis factor α (TNF-α) in vitro (Seo, et al 2002).

H5N1 virus infection begins when the virus enters the host cell after attachment spikes virions to specific receptors on the cell surface from the host. Virions would infiltrate into the cell cytoplasm and will integrate its genetic material in the cell nucleus from the host, and by using the genetic machinery of the cell host, the virus can replicate to form new virions, and these virions can infect surrounding cells. From some results of the examination of clinical specimens taken from patients

with avian influenza H5N1 turned out to replicate in the nasopharynx cells (Peiris et al 2004), and in the gastrointestinal cells (de Jong, 2005 Uiprasertkul, et.al. 2005).

The H5N1 virus can also be detected in the blood , cerebrospinal fluid, and feces of patients (WHO, 2005) . Phase attachment (attachment) is the most crucial phase whether or not the virus can enter the cell host to continue replication. Influenza A virus through hemagglutinin spikes (HA) binds to sialic acid receptors (SA) that exist on the cell host surface. There are important differences between the receptor molecules that exist in humans with receptors that exist in birds or animals . In the bird flu virus , they can recognize and bind to receptors found only on the type of birds which consists of oligosaccharides that containing N-acethylneuraminic acid α-2,3-galactose (SA α -2,3-Gal), which is different molecules with that of the human receptor . Receptors that exist on the surface of human cells is SA -2.6 α-galactose (SA α-2,6-Gal), so theoretically the bird flu virus can not infect humans because of differences in their specific receptors (Radji, 2006).

However, with only one amino acid change in the configuration of these receptors can be altered so that the human receptor can be recognized by the HPAI-H5N1. The potential for the H5N1 virus circulating is what is feared that the virus can create new variants of HPAI-H5N1 thar can be transmitted between humans to humans (Russell & Webster, 2005 Stevensl et al, 2006).

2.5 Control of Avian Influenza Virus

Prevention of avian influenza outbreaks, especially to prevent the transmission to human can be done effectively by increasing the "biosecurity" between fowl and domestic fowl. Many traditionally poultry in Indonesia the level of "biosecurity" is low and in contact with humans, which has been done the people of Indonesia, are ideal for emerging media and the spread of avian influenza. However, the standard implementation of "biosecurity" very strict require huge funds, related with compensation expense to domestic poultry culling and increased standard of "biosecurity" poultry farmers (Niloperbowo, 2013).

The second method is through vaccination of domestic poultry. This method is considered as the most likely way that applied at this time, because it is effective and relatively inexpensive. This vaccine is usually developed to produce a vaccine that can induce defense systems to respond to the presence of Hemagglutinin molecules from avian influenza virus. Antibodies are formed as a result of vaccination will bind Hemagglutinin molecules in the surface of virus particles and eliminates the ability of the virus to initiate infection to the cells. With the standards that are not too tight, the vaccine is made using chicken embryos were inoculated using a variant of avian flu virus were selected. This vaccine production method is a standard method that has been widely adopted by many Indonesian poultry vaccine manufacturers. Indonesia's readiness vaccine companies make Indonesia has a high enough ability to develop avian flu vaccine (Niloperbowo, 2013).

2.6 Overview of Serological Examination

Serological test is a method used to know the antibody titer in the chicken's body. The application of these serological tests are approximately 4 goals, one which is to monitor the results of vaccination. In the process, farmers began to realize the need to perform serological tests especially with the increasing number and types of the disease or vaccination tight schedule. By serological tests, implementation revaccination becomes more precise. In addition, the results of serological tests can also be used as a confirmation of the diagnosis of a disease. Antibody titer of viral diseases such as Newcastle diseases (ND), avian influenza (AI), infectious bursal disease (IBD / Gumboro), infectious bronchitis (IB) and egg drops syndrome (EDS) and bacterial disease that is Korisa, salmonella and chronic respiratory disease (CRD) can be determined by serological tests (Medion, 2008).

2.6.1 Hemaglutination Test

Haemagglutination Test (HA) is used to detect virus which has hemagglutinin. The presence of hemagglutinin will be able to agglutinate erythrocytes of some erythrocytes such as poultry, mammals, and humans. Besides being able to detect the virus that has hemagglutinin, HA test can also be used to measure antigen titer (Indrasari S, 2013). HA titer is the highest dilution that still can agglutinate erythrocytes. HA perfectly characterized by red blood cells coating evenly on the basis of microplate wells and purification of the liquid at the top without the

occurrence of precipitation. While the results showed negative red blood cells shaped point in the middle of the wells (Ernawati et al, 1996; Indrasari, 2013).

2.6.2 Hemaglutination Inhibition Test

Haemagglutination inhibition can be interpreted as a constraint haemaglutination. While haemaglutination is the clumping of red blood cells. Ability to agglutinate is not shared by all the viruses or bacteria that attack chickens but only some viruses and bacteria that have haemaglutinin substances, including paramyxovirus (ND), poxvirus (Pox), adenovirus (EDS), Orthomyxovirus (AI), the bacterium Mycoplasma sp., Haemophilus paragallinarum and Salmonella pullorum. Haemaglutinin substances contained in the body of the virus or bacteria are antigenic to stimulate the formation of specific antibodies. Those formed antibodies have the ability to inhibit blood agglutination caused by viruses or bacteria haemaglutinin (Medion, 2008).

Hemagglutination inhibition test is a serologic tests that prove the formation of specific antibodies hemagglutinin (HA) of H5N1 avian influenza virus from the blood serum. This test can be used to help in the laboratory diagnosis of viral identification, in addition to the HI test can also be used to determine the immune status after vaccination, animals recovering from illness or long term infection disease (WHO 2002; Ernawati et al, 2008; Indrasari, 2013).

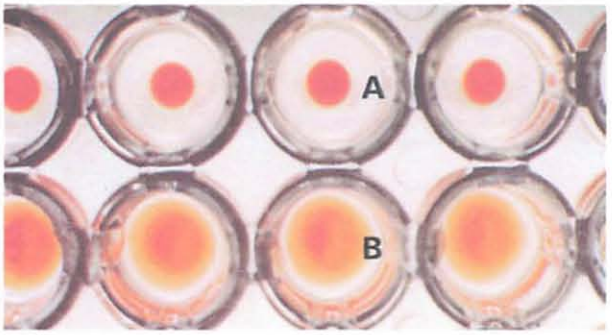

Figure 2.3 : Examples for Hemaglutination Inhibition Test Result showed by the appearance of the agglutination process (A=Aglutination Inhibition), (B=Aglutination Happen).
Source : Medion (2008)

2.6.3 Serum NeutralizationTest

Serum Neutralization Test (SNT) is a sensitive and specific method that can be applied to identify the virus with antibodies specific reactions in humans and animals. The working principle is the reaction of virus neutralization assay with serum containing antibodies then inoculated to the host system such as cell culture or Chicken Eggs Embryonated. If there is no reaction occurs infective virus neutralization reaction is then said to be positive. Serum neutralization against HA influenza virus would inhibit the infection of MDCK cells with a virus. Visualization of virus neutralization activity can be detected in the presence of Cythopathic effect on cells (WHO, 2002; Indrasari, 2013).

Neutralization Test procedure that been used or been known today are the α neutralization test and β neutralization test procedure, serial dilutions of the serum are tested against a standard dose of virus. This technique has certain advantages in that it

use small amounts of serum, is the preferred test for low titered virus. On α neutralization test the virus was serially diluted with standard dose of serum in certain titers (without diluent) (Swayne *et al,* 1998).

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3 MATERIALS AND METHODS

3.1 Research location and Time

This research held at Avian Influenza Research Center (AIRC) with the laboratory Biosafety Level-2 (BSL-2) and laboratory Biosafety Level-3 (BSL-3) Universitas Airlangga. This research was conducted from June to July 2014.

3.2 Material of Research

3.2.1 Research substance

Substance that be used for this Hemaglutination Test is H5 clade 2.1.3 serum stock, PBS (Phosphat Buffer Saline), Chicken RBC (Red Blood Cell) 0,5%, then the substance for Hemaglutination Inhibition Test is a chicken serum, Chicken RBC 0,5%, Phosphat Buffer Saline (PBS) steril, isolate virus H5N1 clade 2.1.3 virus (A/Ck/Ind/114/08), H5N1 clade 2.3.2 virus (A/Dk/Ind/443/12).

Substance for Serum Netralisation Test (SNT) is Cell culture of Madin-Darby Canine Kidney Cells (MDCK) monolayer (konfluen 70-95%), Minimum Essential Medium (MEM lot, RNBC1256), Phospat Buffer Saline (PBS), Fetal Bovine Serum (FBS), L-glutamin, vitamin solution, Bovine Serum Albumin (BSA), Natrium Bicarbonat, antibiotic penicillin-streptomycin, distillated water (DW), formalin 10% and Crystal Violet 2%. H5 clade 2.1.3 serum stock and Antigen H5N1 clade 2.1.3 virus (A/Ck/Ind/114/08), H5N1 clade 2.3.2 virus (A/Dk/Ind/443/12).

3.2.2 Research Equipment

The equipment that be used in this research are Biosecurity Cabinet (BSC), gloves, mask, pippete, vacuum pipette, tray, 1,5 ml eppendorf tube, incubator, yellow tips, blue tips, 96 well "V" microplate, 48 well microtittre plate, 96 well microtittre plate micropipette, laboratory shaker, freezer-80°C (for virus storage), centrifuge, autoclave, waterbath, microscope, T-75 flasks.

3.3 Research Method

3.3.1 Research Sample

The sample that be used in this research is H5 clade 2.1.3 serum stock, H5N1 clade 2.1.3 virus (A/Ck/Ind/114/08), H5N1 clade 2.3.2 virus (A/Dk/Ind/443/12).

3.3.2 Sample Handling in Laboratory

Both serum and antigen was thawed. Furthermore, the serum was tested by hemagglutination inhibition (HI) test by H5N1 clade 2.1.3 virus and then stored at -20 ° C.

3.3.3 Preparation of 0,5% Chicken Erythrocytes Suspension

Erythrocytes which will be used for the hemaglutination inhibition test in this research is 5 ml that were collected in tubes with EDTA anticoagulant (0,1% EDTA / ml of chicken blood). The collected blood then washed with PBS as much as 10 to 15

ml in conical tube. Washing was done by centrifugation at 1500 rpm for 5 minutes. Supernatant was discarded, then add PBS and centrifuge again. This treatment will be done 2-3 times until the supernatant was clear.

Preparation of 10 ml erythrocytes suspension 0,5% as done by :

Formula :

$N_1 \times V_1 = N_2 \times V_2$

$100\% \times V_1 = 0{,}5\% \times 10$ ml

$V_1 = 0{,}05$ ml

$= 50$ µl

Information :

N_1 = Initial Erythrocytes concentration.

V_1 = Initial Erythrocytes Volume.

N_2 = Final Erythrocytes concentration.

V_2 = Final Erythrocytes Volume.

So to make 10 ml of 0,5% erythrocytes suspension is by adding 50 µl Erythrocytes 100% in sterile PBS up to 1000 µl.

3.3.4 Hemaglutination Inhibition Test

The samples tested were H5 clade 2.1.3 serum stock, before testing, the serum should receive special treatment by inactivation to eliminate non-specific substance from serum samples that were able to agglutinate erythrocytes (WHO, 2011). Inactivation performed using Receptor Destroying Enzyme (RDE) with a ratio of

RDE: Serum was 3:1 and incubated overnight at 37 ° C. Then do the heating at a temperature of 56°C for 30 minutes to inactivate the RDE. Six parts of physiological saline (NaCl) was added to obtain a final dilution of 1:10 (Indrasari, 2013).

Step for Hemaglutination Inhibition test begins by inserting 25 µl PBS into each hole microplate V. Serum samples that had been getting treatment inserted into the line A (A1) at 25 µl , and then serial dilutions were made by taking 25 µl of line A no.1 with mixed until uniform , then move to the next hole , and so on until the hole no. 10 . Especially for hole No. 11 is used to control erythrocytes and no. 12 is used to control serum . Furthermore, all of the holes on the line A microplate filled with antigen H5N1 subclade 2.1.3 by 25 µl 4 HAU , except on hole no. 12 4 HAU of antigen was replaced with PBS at 25 µl. Hole no 12 is not filled with the antigen , thus not experiencing erythrocyte agglutination. The absence of agglutination is the case then this point is used as a positive control, whereas the hole no.11 not given antigen in serum but given that erythrocyte agglutination added will have , therefore, this hole is used as a negative control. After the addition of antigen , then incubation at room temperature for 30 minutes. Furthermore, all the holes have been filled by 0.5 % chicken erythrocytes 50 µl, and then incubated at room temperature for 30 minutes. The final step is the determination of the HI titers of antisera were examined. Hemagglutination inhibition reaction occurs if the deposition is formed erythrocytes shaped point in the middle of the wells (Indrasari, 2013; Ernawat *et al*, 2008; WHO, 2002).

3.3.5 Preparation of Cell Culture

MDCK cells cultured in T-75 flasks, one flask enough to grow MDCK cells were 107 cells. The first step is to make the cell culture by pouring medium to the flask and add 5 ml of trypsin-EDTA then shake flask until one minute then dispose of trypsin-EDTA with a pipette. Add 5 ml trypsin-EDTA and shake again for 1 minute and remove with a pipette. Then add 1 ml of trypsin-EDTA averaged on the entire surface of the cells and incubation at 37°C until all cells regardless from the plastic surface (5-10) minutes. Next add 1 ml of FBS to inactivate residual trypsin. Add 8 ml of D-MEM complete and spray gently with a pipette to break up the clot cells. Transfer 10 ml of the mixture in 90 ml of complete D-MEM containing 10% FBS (A mixture of this solution contains about 105 cells / ml). Last take 6 ml of the cell suspension and place it on a T-25 flask subsequently incubated at 37 ° C (WHO, 2002).

3.3.6 Tissue Culture Infective Dose ($TCID_{50}$)

Procedure to test $TCID_{50}$ virus begins by preparing dilutions of 10^{-1} to 10^{-7} in PBS at 315 µl microtube. Furthermore prepare MDCK cells that had been confluent monolayer by 48 well plate . Giving arrows in each well of the A_1 - C_1 with a 10^{-1} mark, A_2 - C_2 with 10^{-2} and so on up to 10^{-7} dilution, whereas the well no 8 to be used as a negative control. Growth Medium discarded then performed three times washing the cells with PBS and PBS in the third wash should be discarded. A total of 100 µl dilution of virus added to each well according to the sign, for negative control virus

was replaced with PBS. Flatten the virus in each well by shake gently and then incubation at 37°C, 5% CO2 for 60 minutes. Plate shaken every 20 minutes. After incubation is complete, virus from each well disposed from negative control to the highest concentration using the same tip. Maintenance medium (MM) included as many as 100 µl per well. Incubation at 37°C, 5% CO2 for 48 hours. After 48 hours, the medium from each well was removed and fixed with 10% formalin gel as much as 100 µl per well , allowed to stand at room temperature for 30-60 minutes. Formalin discarded then washing the cells using flowing water with caution. Then performed using crystal violet staining of cells and leave at room temperature for 5 minutes. Plate was washed under running water. Readings and calculations using the $TCID_{50}$ values Reed and Muench method to calculate 50% positive percentage mortality is more than 50% minus 50% divided by the percentage of positive more than 50% less the percentage is less than 50% positive. Furthermore, the determination of endpoints and add the result to the death of 50% of the endpoints. The results are the outcome of anti log $TCID_{50}$/100µl (Indrasari, 2013 ; WHO, 2002).

3.3.7 Serum Netralisation Test (SNT)

Neutralization test is done by testing serum in quadruplicate then inactivate the serum by means of heating at a temperature of 56°C for 30 min. Then add 60 ml of PBS into each 96 well plate and added with 48 µl PBS on line A (A1-A11). Furthermore insert 12 µl sera that was inactivated in a row (done 4 times replication). Perform 2 times dilution by transferring 60 µl of line A to line B, from B to C, and so

on. Then dispose of 60 µl from row G. The addition of the virus in each well of 60 µl with a concentration of 100 ml in PBS $TCID_{50}$/50 µl on line A to G except the control. In the H line is used for positive control by replacing virus with PBS (Indrasari, 2013).

The next stage doing a back titration which used as proof of 100 TCID50. In this implementation, first set up 8 pieces of 1.5 ml tubes. Then fill the tube first with 438 µl of 100 $TCID_{50}$ of virus, while the second tube to eight filled with 300 µl of PBS. Additionally performed serial dilutions by transferring 138 µl of virus from the first tube to the second tube, 138 µl of virus from the second tube to third tube an so on until the eight tube. Then add 60 µl of PBS from each virus back titration on well and move 60 µl from each well with a dilution back titration four times at the well plate replication. Furthermore slowly shaken well plate and incubated (37°C in a 5% CO2 incubator) for 1 hour (Indrasari, 2013).

After completion of the incubation period, prepare MDCK cells in 96 well plate that had been confluent monolayer. Medium was removed and given 100 ml of PBS to remove residual medium and washing, the washing is done two to three times. Then the mixture was transferred virus and antibodies that have been completed incubated in a well plate containing the cells. MM 100 ml was added and incubated 37°C in a 5% CO2 incubator for 3 days. The examination was conducted using a microscope to determine the presence or absence of CPE (WHO, 2002). Readings can be done based on the results of a back titration (Indrasari, 2013).

3.4 Experimental Design and Data Analysis

Research will be done by taking a serum that had titers of 2^7 antibodi HI test. Because according Risqiawan (2011) That titer serum capable to neutralizing AI virus. Furthermore virus $TCID_{50}$ values taken by using the Reed-Muench method. Then proceed with the SNT method on both kinds of virus, H5N1 clade 2.1.3 to see the ability to neutralize the virus and the H5N1 clade 2.3.2 to see the cross reaction. SNT method was carried out in accordance Reed-Muench calculation.

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Reed and Muench Formula

$$PD = \frac{(percentage\ positif\ above\ 50\%) - 50\%}{(percentage\ positif\ above\ 50\%) - (percentage\ positif\ below\ 50\%)}$$

Log 50% endpoint = (log dilution above 50%) – (PD x log dilution factor)

$TCID_{50}$ calculations by adding PD factor to the dilutions that showed> 50% positive. If the well was inoculated with 0.1 $TCID_{50}$ of the virus dilution can be written by $TCID_{50}$ / 0.1 ml (WHO, 2002).

3.6 Flow Chart

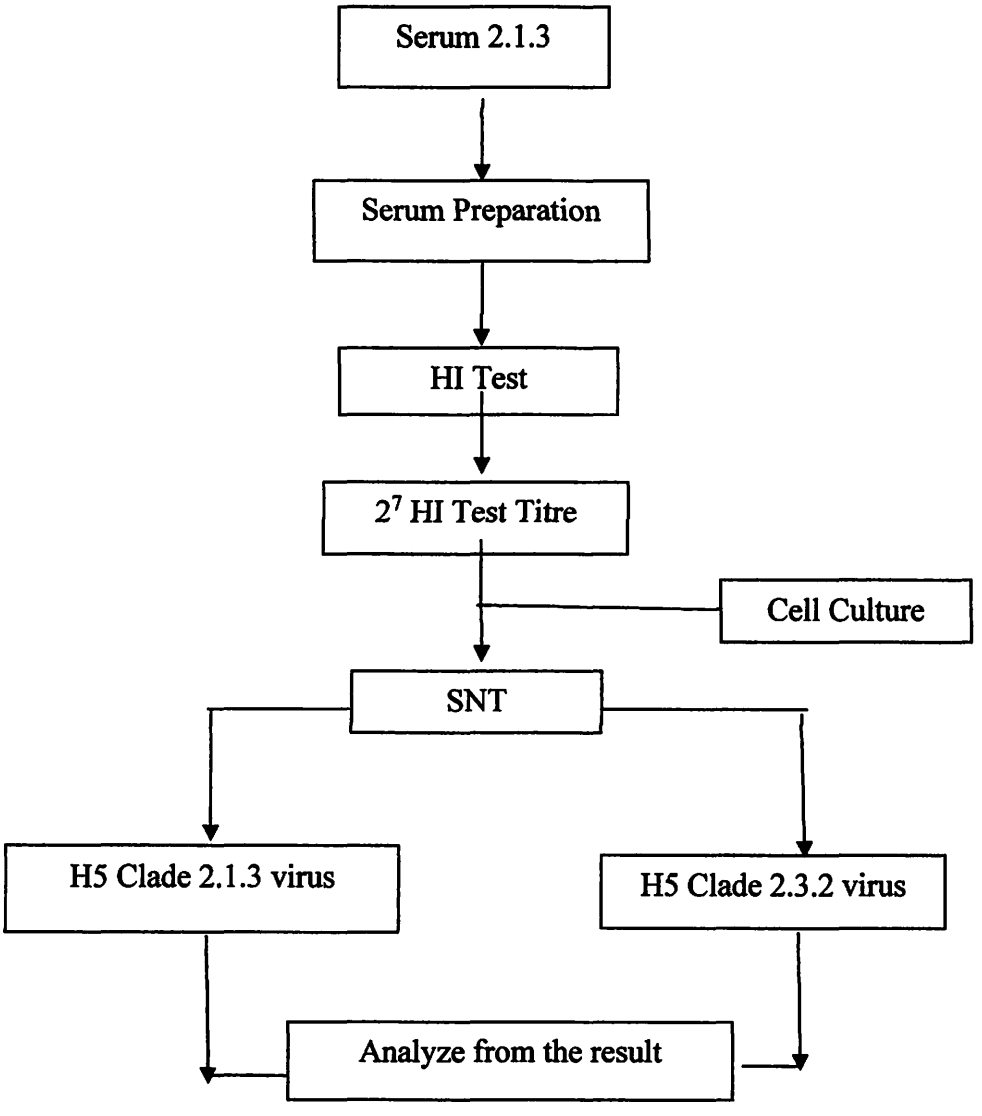

Figure 3.1 : Flow Chart of Research

CHAPTER 4

RESULT

CHAPTER 4 RESULT

The H5 clade 2.1.3 serum stock of AIRC Universitas Airlangga got titre of 2^7 in the HI test. Virus stock of H5 clade 2.1.3 (A/Ck/Ind/114/08) and clade 2.3.2 (A/Dk/Ind/443/12) was calculated to find $TCID_{50}$. The SNT was performed by those result.

$TCID_{50}$ and SNT result of this research will be presented in the table.

4.1 Calculation of Virus Titre

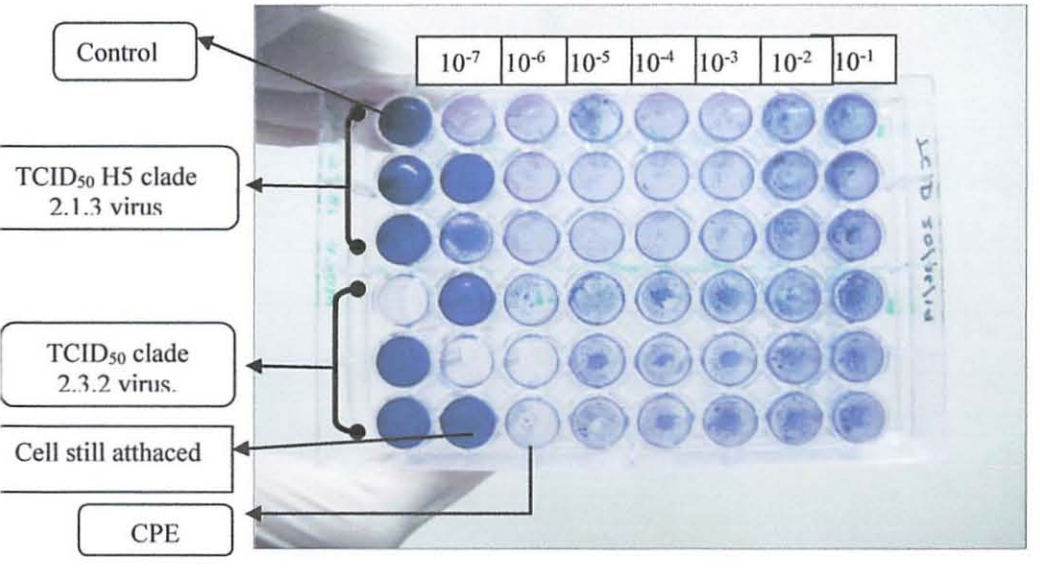

Figure 4.1 TCID Result

The $TCID_{50}$ of H5 clade 2.1.3 virus (A/Ck/Ind/114/08) and clade 2.3.2 (A/Dk/ind/443/12) can be seen in the figure 4.1 above. Three rows from the top to Eight columns to the right was $TCID_{50}$ result of H5 clade 2.1.3 virus and the remnant was

was $TCID_{50}$ result of H5 clade 2.3.2 virus. Blue print in the wells shows that the cells still attached in the well plate. And the plain one shows CPE.

The $TCID_{50}$ of H5 virus clade 2.1.3 (A/Ck/Ind/114/08) and clade 2.3.2 (A/Dk/ind/443/12) result was showed in the table below.

Table 4.1.a $TCID_{50}$ result of H5 clade 2.1.3 virus

Virus Dilutions	CPE		Total		Ratio (+)	% positive (+)
	+	-	+	-		
10^{-1}	3	0	20	0	20/20	100
10^{-2}	3	0	17	0	17/17	100
10^{-3}	3	0	14	0	14/14	100
10^{-4}	3	0	11	0	11/11	100
10^{-5}	3	0	8	0	8/8	100
10^{-6}	3	0	5	0	5/5	100
10^{-7}	2	1	2	1	2/3	67
Control	-	3	-	-	-	-

$TCID_{50}$ of H5N1 virus (A/Ck/Ind/114/08) is 1.78×10^8 $TCID_{50}$/ml. The result was calculated by Reed and Muench formula (appendix 1).

Table 4.1.b $TCID_{50}$ result of H5 clade 2.3.2 virus

Virus Dilutions	CPE		Total		Ratio (+)	% positive (+)
	+	-	+	-		
10^{-1}	3	0	19	0	19/19	100
10^{-2}	3	0	16	0	16/16	100
10^{-3}	3	0	13	0	13/13	100
10^{-4}	3	0	10	0	10/10	100
10^{-5}	3	0	7	0	7/7	100
10^{-6}	3	0	4	0	4/4	100
10^{-7}	1	2	1	2	1/3	33
Control	-	3	-	-	-	-

$TCID_{50}$ of H5N1 virus (A/dk/Ind/443/12) is 5.62×10^7 $TCID_{50}$/ml. The result was calculated by Reed and Muench formula (Appendix 2).

4.2 Serum Neutralization Test

Table 4.2 SNT result of H5 clade 2.1.3 serum to H5 clade 2.1.3 virus

Serum dilution		CPE		Total		Ratio (+)	% (+)	% protective
Quantitatve	Log_{10}	+	-	+	-			
1:10	10^{-1}	0	4	0	18	0/18	0	100
1:20	$10^{-1.3}$	0	4	0	14	0/14	0	100
1:40	$10^{-1.6}$	0	4	0	10	0/10	0	100
1:80	$10^{-1.9}$	0	4	0	6	0/6	0	100
1:160	$10^{-2.2}$	2	2	2	2	2/4	50	50
1:320	$10^{-2.5}$	4	0	6	0	6/6	100	0
1:640	$10^{-2.8}$	4	0	10	0	10/10	100	0
Control		0	4	-	-	-	-	

The result of H5 clade 2.1.3 serum to H5 clade 2.1.3 virus is positive (+). The serum was able to neutralize the virus and protect cell culture. The 50% endpoint of neutralization of the serum is 1:160. It means that the serum can protect at least 50% of the cells by neutralizing the virus at the 1:160 dilutions.

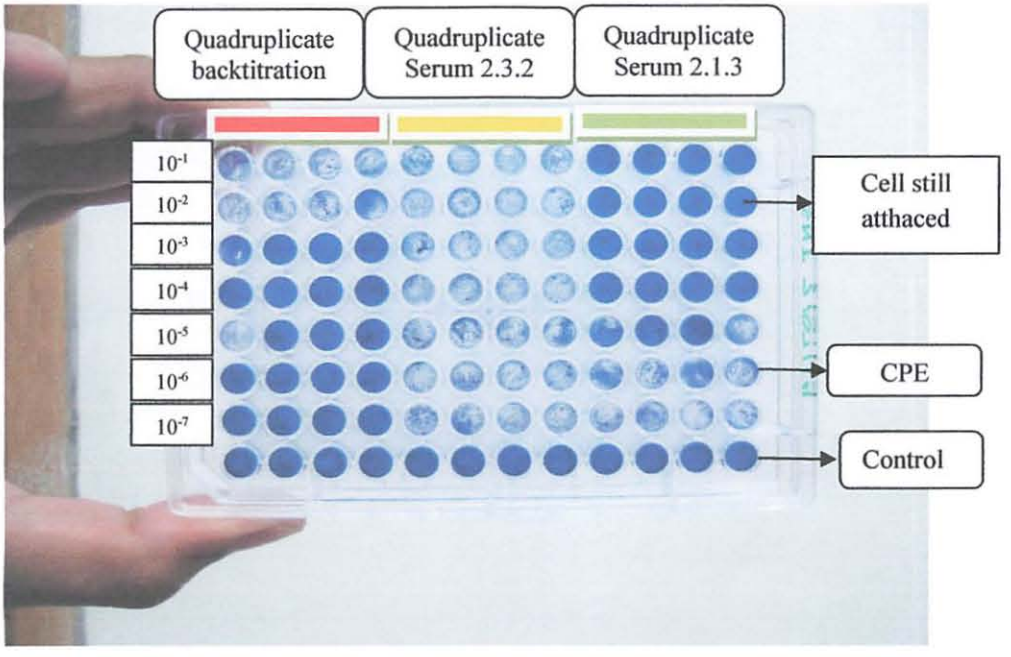

Figure 4.2 SNT result of H5 clade 2.1.3 serum to 2.1.3 virus (green ribbon)

Table 4.3 SNT result of H5 clade 2.1.3 serum to H5 clade 2.3.2 virus

Serum dilution		CPE		Total		Ratio	% (+)	% protective
Quantitatve	Log_{10}	+	-	+	-	(+)		
1:10	10^{-1}	0	4	0	27	0/27	0	100
1:20	$10^{-1.3}$	0	4	0	23	0/23	0	100
1:40	$10^{-1.6}$	0	4	0	19	0/19	0	100
1:80	$10^{-1.9}$	0	4	0	15	0/15	0	100
1:160	$10^{-2.2}$	0	4	0	11	0/11	0	100
1:320	$10^{-2.5}$	0	4	0	7	0/7	0	100
1:640	$10^{-2.8}$	1	3	1	3	1/4	25	75
control		0	4	-	-	-	-	

The result of H5 clade 2.1.3 serum to H5 clade 2.3.2 virus is positive (+). The serum was able to neutralize the virus and protect cell culture. The 50% endpoint of neutralization of the serum is 1:1258. It means that the serum can protect at least 50% of the cells by neutralizing the virus at the 1:1258 dilutions. The result was calculated by Reed anf Muench formula (Appendix 3).

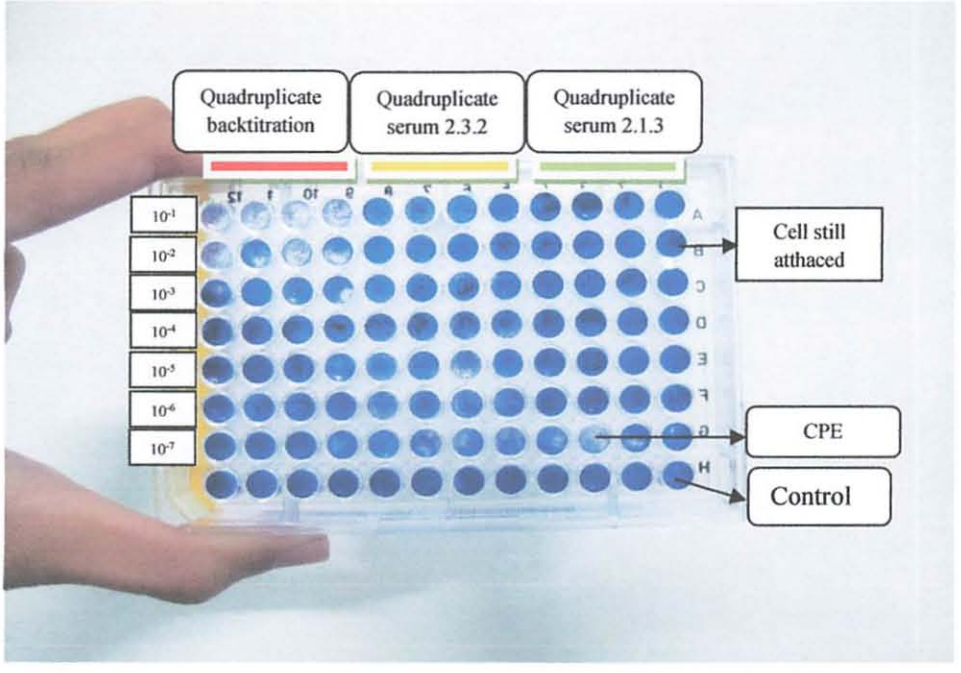

Figure 4.3 SNT result of H5 clade 2.1.3 serum to 2.3.2 virus (green ribbon)

CHAPTER 5

DISCUSSION

CHAPTER 5 DISCUSSION

In this research, the result of the $TCID_{50}$ of the H5 clade 2.1.3 virus (A/Ck/ind/114/08) is 1.78 x 10^8 $TCID_{50}$/ml and clade 2.3.2 virus (A/Dk/Ind/443/12) is 5.62 x 10^7 $TCID_{50}$/ml. Based on this result showed that the clade 2.1.3 virus more pathogen than clade 2.3.2 virus in MDCK. This result was calculated by Reed and Muench formula. The principle of $TCID_{50}$ is to calculate virus dose that required to infect 50% of cell culture (Swayne et al., 1998). It characterized by Infectious $Dose_{50}$ (ID_{50}) for the level of infection that occurs through serial dilutions of virus and inoculated on MDCK cells. Calculation of the infection result can be seen 72 hours post inoculation. It was characterized by the presence of CPE on MDCK cells that had been stained (WHO, 2011). The $TCID_{50}$ result showed that the suspension of H5 clade 2.1.3 virus (A/CK/Ind/114/08) and clade 2.1.3 (A/Dk/Ind/443/12) is feasible to be used as a test virus.

The HI titre of serum antibody is 2^7 and be used on the SNT. Minimum HI serological titres in field birds should be 1/32 to protect from mortality or greater than 1/128 to provide reduction in challenge virus replication and shedding (OIE, 2015). Neutralization assay is an assay that is highly sensitive and specific in identifying antigen / antibody and that is able to know the neutralize ability of an antibody to antigen. The interpretation of SNT result was expressed by the level of cell damage (CPE) that occurs after serum-antigen mix were inoculated in the cell culture.

culture. The positive result indicates that serum containing specific antibody which can inhibit virus infection on MDCK cells (WHO, 2011).

The result of SNT showed that the serum were able to neutralize and protect at least 50% of cell culture at the 1:160 dilution to H5 clade 2.1.3 virus. It is consistent with the research result of Risqiawan (2011) that said the antibody titre of the serum which able to neutralize and give protection if it has 2^7 of HI titre. While serum tested with H5 clade 2.3.2 virus on SNT, it was showed that there were cross reaction, the serum was able to neutralize the virus and protect at least 50% of cell culture at the 1:1258 dilutions to H5 clade 2.3.2 virus. The antibody 2.1.3 more protective to clade 2.3.2 virus than clade 2.1.3 virus.

Based on this result showed that the antibody clade 2.1.3 could protect both of the viruses, that means the antibody clade 2.1.3 had wide range of compatibility. Wibowo (2014) said, The SNT result shows that H5 clade 2.3.2 vaccine was able to neutralize H5 clade 2.3.2 virus (A/Dk/Ind/443/12). But it was not able to neutralize H5 clade 2.1.3 virus (A/Ck/Ind/114/08).

That conditions can occur because the antibody 2.1.3 which produce from the vaccine have immunogenic side more compatible with the clade 2.3.2, because we did not compare between the vaccine which available in Indonesia that there were a different content of the massa antigen and adjuvant that could make the reaction occurred (Swayne *et al*, 2006 ; Dharmayanti *et al*, 2015).

Beside that, Avian Influenza virus has a characteristic easily to mutation (Dharmayanti and Hewajuli, 2008) so it was possible there were changes flipping from 2.3.2 virus and changes structure of the amino acids that could make antigenic reaction to be compatible with the antibody clade 2.1.3. By observing the changes of the amino acids in the antigenic site, which also represents the location of antigenic epitope binding, the immune response could be observed to be different (Setyawati *et al*, 2011).

It is consistent with the research result of Dharmayanti *et al* (2013), showed the homology between virus clade 2.3.2 with clade 2.1.3 on the level nukloetide is 92%, whereas the amino acid is 89-90%. Clade 2.3.2 virus has the amino acid multi basic the HA protein, but there were differences in cleavage site of the HA protein. And when compared the virus clade 2.1.3 the deletion of an acid amino at position -6HA (PQRERRRKR // G) shows that no amino acid is located at surface proteins or antigenic site of virus H5N1 clade 2.1.3, so it is unlikely will lead to changes in antigen-antibody recognition. Results of these studies shows, AI virus H5N1 clade 2.1.3 can be used to control the H5N1 virus clade 2.3.2 (Dharmayanti *et al*, 2015).

According to this research, the use of Avian Influenza vaccine from clade 2.1.3 has been able to protect susceptible animals against both avian influenza virus clade 2.1.3 and clade 2.3.2 infection. So, it is better to choose the vaccine that compatible with the virus in field.

CHAPTER 6

CONCLUSION AND SUGGESTION

CHAPTER 6 CONCLUSION AND SUGGESTION

6.1 Conclusion

There were cross reaction between avian influenza virus clade 2.1.3 and 2.3.2 to 2.1.3 clade avian influenza serum by Serum Neutralization Test. The serum of H5 clade 2.1.3 was able to neutralize H5 clade 2.1.3 virus. It can neutralize and protect at least 50% of culture cell in the SNT at 1:160 dilutions. While serum tested with H5 clade 2.3.2 virus on SNT, it was able to neutralize the virus and protect at least 50% of cell culture at the 1:1258 dilutions.

6.2 Suggestion

1. Surveillance should be done in order to know the evolution of H5N1 virus in Indonesia.
2. Actually seed vaccine clade 2.1.3 can be used to protect susceptible animals against both avian influenza virus clade 2.1.3 and clade 2.3.2 infection.

SUMMARY

SUMMARY

Muhammad Khaliim Jati Kusala, Cross Reactivity Between H5 Clade 2.1.3 Serum and H5 Clade 2.3.2 Virus by Using Serum Neutralization Test. This Research was conducted under guidance of Prof. Dr. Chairul Anwar Nidom, M.S., drh. as supervisor and Prof. Dr. Setiawan Koesdarto, MSc., drh as co supervisor.

Avian Influenza is belonged to the H5N1 subtype of Influenza A virus which can infect humans and many other animal species. Because the characteristics of the influenza virus constantly evolve and change unpredictably, it is very important to control the virus. But to choose the right vaccine strain, has many challenges and sometimes lead to suboptimal protection. Moreover, predicting the next pandemic virus, including when and where will arise, is currently difficult.

The Aim of this research is to know the cross-reactions between avian influenza virus clade 2.1.3 and 2.3.2 to 2.1.3 clade avian influenza serum by using serum neutralization test.

The method of this research is using H5 clade 2.1.3 serum stock AIRC Universitas Airlangga that be tested against H5 clade 2.1.3 (A/Ck/Ind/114/08) and 2.3.2 (A/Dk/Ind/443/12) virus by using Serum Neutralization Test assay. The serum that be used has 2^7 of HI titre. The TCID50 of the viruses are 1.78 x 108 TCID50/ml of H5 clade 2.1.3 and 5.62 x 107 TCID50/ml of H5 clade 2.3.2.

The result of SNT showed that the serum were able to neutralize and protect at least 50% of cell culture at the 1:160 dilution to H5 clade 2.1.3 virus. While serum

tested with H5 clade 2.3.2 virus on SNT, it was showed that there were cross reaction, the serum was able to neutralize the virus and protect at least 50% of cell culture at the 1:1258 dilutions to H5 clade 2.3.2 virus.

According to this research, the use of Avian Influenza vaccine from clade 2.1.3 has been able to protect susceptible animals against both avian influenza virus clade 2.1.3 and clade 2.3.2 infection.

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APPENDIX

Appendix

Appendix 1. Procedure of $TCID_{50}$ H5 clade 2.1.3 virus

a. Proportional Distance

$$PD = \frac{\text{(percentage positive above 50\%)} - 50\%}{\text{(percentage positive above 50\%)} - \text{(percentage positive below 50\%)}}$$

$$= \frac{67\% - 50\%}{67\% - 0\%}$$

$$= \frac{17}{67}$$

$$= 0.25$$

b. 50% endpoint

$$50\%\ DEP = 10^{-7-0.25}$$

$$= 10^{-7.25}$$

c. Log $TCID_{50}$

$$\text{Log } TCID_{50} = \log 10^{-7.25}$$

$$= 1.78 \times 10^{-7}\ TCID_{50}/100\ \mu l$$

By 1.78×10^{7} times of 100 µl virus dilution will kill at least 50% cell culture.

d. $TCID_{50}$/ml

$$TCID_{50}/ml = 1.78 \times 10^{-8}$$

Appendix 2. Procedure of $TCID_{50}$ H5 clade 2.3.2 virus

a. Proportional Distance

$$PD = \frac{\text{(percentage positive above 50\%)} - 50\%}{\text{(percentage positive above 50\%)} - \text{(percentage positive below 50\%)}}$$

$$= \frac{100\% - 50\%}{100\% - 33\%}$$

$$= \frac{50}{67}$$

$$= 0.75$$

b. 50% endpoint

$50\%\ DEP = 10^{-6-0.75}$

$= 10^{-6.75}$

c. Log $TCID_{50}$

Log $TCID_{50} = \log 10^{-6.75}$

$= 5.62 \times 10^{-6}\ TCID_{50}/100\ \mu l$

By 5.62×10^{6} times of 100 µl virus dilution will kill at least 50% cell culture.

d. $TCID_{50}$/ml

$TCID_{50}/ml = 5.62 \times 10^{-7}$

Appendix 3. Procedure of 50% endpoint SNT of H5 clade 2.1.3 serum and H5 clade 2.3.2 virus

a. Proportional Distance

$$PD = \frac{\text{(percentage positive above 50\%)} - 50\%}{\text{(percentage positive above 50\%)} - \text{(percentage positive below 50\%)}}$$

$$= \frac{75\% - 50\%}{75\% - 0\%}$$

$$= \frac{25}{75}$$

$$= 0.33$$

b. Log 50% endpoint

Log 50% DEP = (log dilution above 50%) – (PD x log dilution factor)

$= \text{Log } 10^{-2.8} - (0.33 \times 1)$

$= 2.8 - 0.5$

$= 3.1$

c. 50% endpoint neutralization

50% endpoint neutralization $= 10^{-3.1}$

$= 1:1258$

The serum can protect at least 50% of the cells by neutralizing the virus at the 1:1258 dilutions

Appendix 4. Research equipment, substance and process

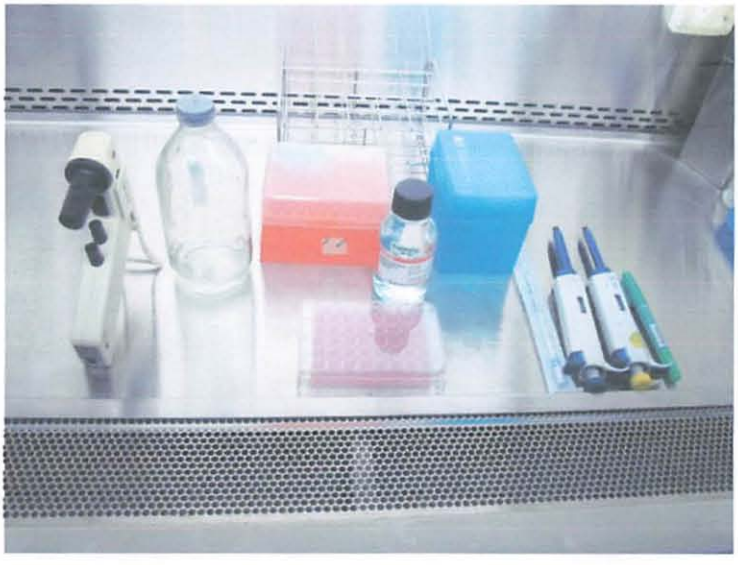

Figure 1 : SNT assay equipment

Figure 2 : Fixation process

Figure 3 :Test $TCID_{50}$

Figure 4 : Incubation at 37°C, 5% CO_2.

Figure 5 : Staining process