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Purification and characterization protein of anti-dengue specific immunoglobulin Y for diagnostic kit of dengue

[1]Sri Agus Sudjarwo; [2]Koerniasari Eraiko Sudjarwo; [3]Koerniasari
[1]Faculty of Veterinary Medicine Airlangga University, Indonesia.
[2]Faculty of Dental Medicine, Airlangga University, Indonesia.
[3]Study Program of Environmental Health, Polytechnic of Health, Surabaya 60115, Indonesia.

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ABSTRACT

This study aimed to produce immunoglobulin Y (IgY) specific to dengue virus which could be used for diagnostic kit of dengue. Lohman laying hens were immunized intramuscularly with antigenic of dengue. Egg yolk was separated from egg white and IgY antibody was then purified by multiple polyethylene glycol (PEG) 6000 extraction and ammonium sulfate purification steps. The IgY concentration in egg yolk increased during the immunization period until week 6 where it began to increase dramatically at 2 weeks and it reached a plateau at 4 weeks after immunization. After week 6 the levels decreased gradually. Antibody of dengue was detected and produce a specific line of precipitation in agar gel precipitation test (AGPT) beginning the second week after the first immunization. Analysis of results obtained with ELISA showed significant increase in the dengue-specific antibodies after two weeks from the primary immunization. Through the effect of boostering; the anti-dengue antibody levels reached a plateau at four weeks from the primary immunization and remained significantly higher till the end of observation period. SDS-PAGE revealed the IgY preparation to be pure and dissociated into protein bands with molecular weights of 145; 66; 45, 33 and 26 kDa and western blot analysis revealed the presence of anti-dengue IgY in egg yolks protein, with a molecular weights of approximately 66 kDa. These results suggested that chicken IgY could be a practical strategy in large-scale production of specific anti-dengue Ig Y for diagnostic KIT of dengue.

INTRODUCTION

Dengue is an arthropod-borne flavivirus that comprises four distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) that constitute an antigenic complex of the genus flavivirus, family Flaviviridae. Dengue virus infections can result in a range of clinical manifestations from asymptomatic infection to dengue fever (DF) and the severe disease dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Most dengue infections are asymptomatic or cause mild symptoms, which are characterized by undifferentiated fever with or without rash. Typical DF is characterized by high fever, severe headache, myalgia, arthralgia, retro-orbital pain and maculopapular rash. Some patients show petechiae, bruising or thrombocytopenia (Kurane, 2007). The dramatic increase in the global burden of dengue has spurred increased public and private sector interest in developing improved diagnostics for dengue infections. Immunoglobulins are widely used for a variety of purposes, such as in diagnostic tests, purification columns, and passive immunotherapy (Arasteh *et al.,* 2004; Chalghoumi *et al.,* 2009). Therefore, research and diagnostic community constantly demand new alternatives and procedures to produce cost-effective antibodies. The use of laying hens to produce polyclonal antibodies is an alternative to the use of mammals, such as rabbits and, since more than two decades, egg yolk antibodies (IgY) are a low cost and ethical alternative (Schade *et al.,* 2005; Rahimi *et al.,* 2007; Pauly *et al.,* 2009). Compared with the stressful bleeding of mammals to obtain serum, IgY can be easily obtained non-invasively from the egg yolk. From the economical point of view, the amount of antibodies produced bya single hen is similar to that of a largemammal such as a sheep or goats, whereas maintenance costs are much lower (Fu *et al.,* 2006; Schade *et al.,* 2005). IgY from serum is actively transferred into the yolk by a receptor-

** Corresponding Author*
Prof. Sri Agus Sudjarwo.,Ph.D
Department of Pharmacology, Faculty of Veterinary Medicine, Airlangga University, Surabaya 60115, Indonesia. Tel: +62-31-5992785;

mediated process and the amount of the immunoglobulin varies between 100 and 250 mg per egg (Schade *et al.,* 2005). Thus, a substantial amount of antibody can be produced from just one hen (up to 40 g of total IgY per chicken per year), of which 1-10% is expected to be specific to the antigen of interest (Mine and Kovacs- Nolan, 2002). In contrast to mammalian IgG, IgY antibodies do not activate mammalian complement, do not cross-react with Fc receptors, mammalian rheumatoid factor, or human anti-mouse antibodies, thus eliminating false-positive results in serological assays (Schade *et al.,* 2005; Alexander *et al.,* 2009). Also, chickens are able to develop a better response against mammalian antigens, due to the phylogenetic distance between mammals and birds (West *et al.,* 2004; Schade *et al.,* 2005). There is an increasing interest in the use of chicken egg yolk for polyclonal antibody production for practical and economical reasons (Zhen *et al.,* 2009; Wang *et al.,* 2011) and chicken egg yolk antibodies (IgY) have been applied successfully for scientific, diagnostic, prophylactic and therapeutic purposes (Lee *et al.,* 2009; Liou *et al.,* 2010; Sui *et al.,* 2011). Because of the phylogenetic distance between birds and mammals, mammalian proteins are often more immunogenic in birds than in other mammals and antibody synthesis readily stimulated in hens (Leslie and Clem 1969). In addition, because of the phylogenetical distance bird antibodies against a mammalian protein may often react with analogous proteins in other mammalian species (Alexander *et al.,* 2009; Lu *et al.,* 2009).

This study aimed to produce IgY specific to dengue virus which could be used for future alternative diagnostic kit, prophylactic and therapeutic medicines for dengue.

MATERIALS AND METHODS

Preparation of viral antigen

Dengue virus of the D2 strain was obtained from the Institute of Tropical Disease Airlangga University (Surabaya, Indonesia) and grown for 72 h at 34°C in the allantoic fluid of 9-day-old chicken embryos. The virus was harvested and cleaned by centrifugation at 2000 x g, 40°C for 20 min, then concentrated with 8% PEG-6000 (Fluka AG, Buchs, Switzerland). The mixture was stirred at 4°C overnight and centrifuged at 5000 x g, 10oC for 20 min. The pellet containing the virus was resuspended in tris-buffered saline (TBS) (10 mM Tris, 150 mM NaCl, pH 7.5) and dialyzed against this solution at 4°C for 24h. The virus was then inactivated by treatment with 0.05% (v/v) formaldehyde at 4°C for 24h. This viral sample was used to immunize the hens.

Immunization of hens with dengue virus

Lohman laying hens were immunized intramuscularly with dengue virus that had been inactivated using formaldehyde at 4°C for 24h. The immunizations were repeated four times with dose of each 80 µg of antigen (viral protein) of dengue with an interval of one week. The first immunizations were antigen mixed with Fruend Adjuvant Complete and subsequently mixed with Freund Adjuvant Incomplete.. Eggs were collected daily, beginning before and after the first immunizations, and stored at 4°C until analysis.

Isolation and purification of IgY

A rapid and simple method adapted from previous studies (Almeida *et al.,* 2009) was used to extract IgY from yolk. Briefly, the yolk was separated from the white by egg separators, and a volume of buffer containing 14% PEG6000 (w/v) equivalent to three volumes of yolk was added. The mixture was stirred at room temperature (RT) for 30 min and was centrifuged at 5000g for 20 min at 10 ^{o}C. The supernatant was collected and filtered through four layers of sterile gauze. The volume of the filtrate was measured, and PEG6000 was added by gentle stirring to adjust the final polymer concentration to 12% (w/v). The material was centrifuged at 5000g for 20 min at 10 ^{o}C. The pellet was dissolved to the original volume of yolk in phosphate buffer, solid ammonium sulfate was added to reach 50% saturation, and the mixture was stirred overnight at 4 ^{o}C. The precipitate was collected by centrifugation and washed with 33% saturated ammonium sulfate. The precipitate was dialyzed against PBS and freeze-dried, and the powder obtained was stored at -20 ^{o}C. The purified IgY concentration in egg yolk determined by spectrophotometer (Biorad,USA) and Bradford method. Finally, the IgY antibodies were stored at -20ºC until use (15).

Agar gel precipitation test (AGPT)

AGPT was carried out on the imumunoglobulin Y samples using the methods described by Okworl (2011) It was performed using immunodiffusion plates with 10 ml of 1% agar noble gel containing 8% sodium azide at PH 7.2 ± 0.1. Using a template and cutter wells of 4 mm diameter and 4 mm interspace (apart) were cut, the plates were set up with groups of 6 wells in a circle surrounding a centre well. The peripheral wells were filled with the immunoglobulin Y samples to be tested, while the centre well was filled with the antigen. The plates were incubated at 37°C and read at 24, 48, and 72 h under diffused light. The observations were compared with before and after immunization. Positive IgY antidengue samples showed a line of precipitation between the IgY and antigen wells, while negative IgY ntidengue samples showed no line of precipitation.

Enzyme-linked immunosorbent assay

The titer of IgY against dengue virus was measured by an indirect noncompetitive enzyme-linked immunosorbent assay (ELISA) according to previously reported methods with modifications (Zhen *et al.,* 2009). A 96-well micro-titer plate was coated with inactivated dengue virus containing 0.58 mg/mL protein in carbonate–bicarbonate buffer (0.05 M, pH 9.6) at 100 uL/well. Serial dilutions of specific IgY were incubated in the pre-coated and blocked plate for 1 h before the bound IgY was detected with 100 uL/well HRP-conjugated rabbit anti-chicken IgY (1:5000) (Promega, USA). After incubation for 1 h at 37 ^{o}C, the plate was washed four times with PBS containing 0.05% Tween 20 (PBST). Next, 100 uL/well 3,30-5,50-

tetramethylbenzidine (Amresco, USA) substrate was added and incubated for 15 min at 37 °C. The color development was stopped with 2 M sulfuric acid (50 uL/well), and the optical density (OD) was measured on a micro-titer plate reader (Tecan f200, Switzerland) at 450 nm. The reproducibility of the experiment was ascertained by including a blank control (PBS) and a negative control (IgY derived from non-immunized hens) in each plate. IBV-specific IgY titer was defined as the maximum dilution multiple of the sample with an OD value that was 2.1 times that of the negative control.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to determine the purity of IgY. A 15% polyacrylamide gel was used with a Mini-PROTEAN 3 cell (Bio-Rad Laboratories, USA). The analysis was conducted under reducing conditions; the sample was mixed with sample buffer and held for 5-8 min at 100 °C. Ten microliters of the sample was loaded into each well. Pre-stained protein standard (Fermentas, Lithuania) was used as a molecular weight marker. The protein bands were visualized with Coomassie Brilliant Blue R250 (Fluka USA). The gel was analyzed using Bio-Rad image analysis software.

Western blotting assay

To confirm the specificity of anti-dengue IgY to dengue virus proteins, western blotting was conducted using a previously published method with some modifications (Qiu *et al.,* 1992). Briefly, 100 uL of purified dengue virus containing 1-2 mg/mL protein, as determined by the Bradford method, was mixed with 100 uL of electrophoresis sample buffer. The disrupted virus preparation was subjected to SDS-PAGE in a 14% slab polyacrylamide gel separated by a 4% stacking gel at 100 V for 3.5 h at RT. After electrophoresis, the gel was equilibrated in transfer buffer for 15-30 min, after which the viral proteins were electrically transferred onto a nitrocellulose (NC) membrane (Osmonics, USA) for 1 h at 350 mA at 4 °C. The NC membrane was cut into 0.5-cm strips, which were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% non-fat dry milk for 1 h at RT and was incubated overnight at 4 °C with a 1:100 dilution of specific or nonspecific IgY. After incubation, the strips were washed four times with PBS-T and incubated with HRP conjugated rabbit anti-chicken IgY (Promega, USA) diluted 1:1000. Following 2 h of incubation at RT, the strips were washed three more times. After washing, the strips were incubated in developing buffer containing 4 mg 3,30-diaminobenzidine tetrahydrochloride (Aladdin, China) in 5 mL Tris-HCl and 15 lL hydrogen peroxide for 3-5 min. This reaction was stopped by rinsing with distilled water. The strips were photographed after development.

Statistical analysis

Data were reported as means ± standard deviation (SD) and levels of significance were evaluated using one-way ANOVA with Least Significant Difference test. The differences were considered significant at the level of $p < 0.05$.

RESULTS

The IgY concentration in egg yolk.

The IgY concentration in yolk from eggs before immunizations was 4.20 ± 0.34 mg/ml of egg yolk. Whereas the IgY concentration in egg yolk demonstrated significantly increased beginning the second week after the first immunization 4.81 ± 0.42 mg/ml and reached maximum of 5.77 ± 0.59 mg/ml at 4 week after immunization. After week 6 the levels decreased gradually to reach a level of 5.43 ± 0.54 mg/ml (Table 1).

Table. 1: Concentration of immunoglobulin Y (IgY) content before and after immunization

Group	Concentration of IgY(mg/ml) X ± SD
Before immunization	4.20 ± 0.34a
At 2 weeks after immunization	4.81 ± 0.42 b
At 4 weeks after immunization	5.77 ± 0.59 c
At 6 weeks after immunization	5.43 ± 0.54 d

The data represent the average from 15 eggs.
Superscript within each column indicate significant difference between the means ($p < 0.05$).

Agar gel precipitation test of IgY

AGPT reactions between dengue antigen and IgY before or after immunization are shown in Figure 1. IgY positive anti dengue if the precipitation lines from the IgY into the well. One line of precipitation was produced by IgY from 2; 4 and 6 week after immunization but not before immunization. The AGPT test was a satisfactory and uncomplicated technique for detecting precipitating antibodies against dengue virus.

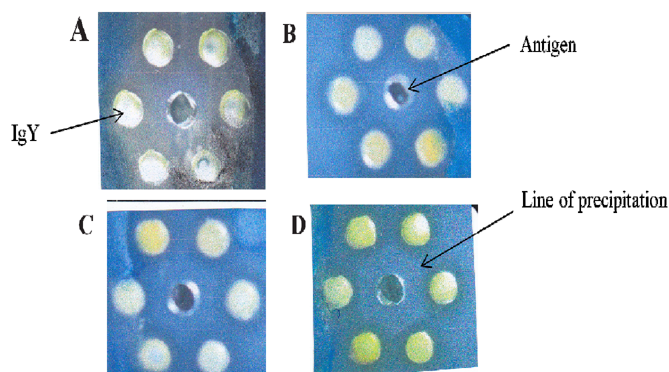

Fig. 1: AGPT reactions between dengue antigen and IgY before immunization (A); at 2 weeks (B); 4 weeks (C) and 6 weeks (D) after immunization

IgY titres off egg yolk antibodies with specificity against dengue virus.

Specific dengue IgY were detected at 2 week after immunization. Following reimmunization, the level of specific antibodies continued to increase at 4 and 6 week after immunization. 2; 4 and 6 weeks after immunization demonstrated significantly higher egg yolk antibody titers than that of before immunization (Table 2).

Table. 2: Titer of anti-dengue IgY in egg yolks were measured by ELISA.

Group	Titer of anti-dengue IgY (OD) X ± SD
Before immunization	0.48 ± 0.12 [a]
At 2 weeks after immunization	2.42 ± 0.28 [b]
At 4 weeks after immunization	3.79 ± 0.24 [c]
At 6 weeks after immunization	3.56 ± 0.25 [d]

The data represent the average from 15 eggs.
Superscript within each column indicate significant difference between the means ($p < 0.05$).

Characterization of IgY by SDS-PAGE

SDS-PAGE was applied to confirm isolation of immunoglobulin Y. Analysis of purified IgY by SDS-PAGE identified two major bands of 66 and 26 kDa, and three minor bands of 145; 45 and 33 kDa. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing 66 kDa heavy chain and 26 kDa light chain. (Figure 2).

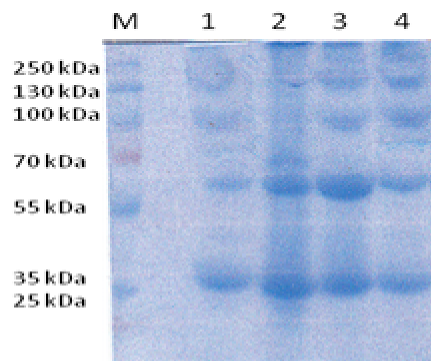

Fig. 2: SDS–PAGE of IgY. Protein markers (M). IgY before immunization (Lane 1). IgY after immunization at 2 weeks (Lane 2); 4 weeks (Lane 3) and 6 weeks (Lane 4).

Characterization of IgY by Western blotting

The presence of specific anti-dengue IgY in egg yolks and its molecular weight were determined by Western blotting, were a single protein band of molecular weight protein (66 KDa) showed the presence of specific anti dengue IgY (Figure 3). These stronger band were detected in egg yolks of hens from 2; 4; and 6 weeks after immunization, while in egg yolks of hens before immunization was undetectable.

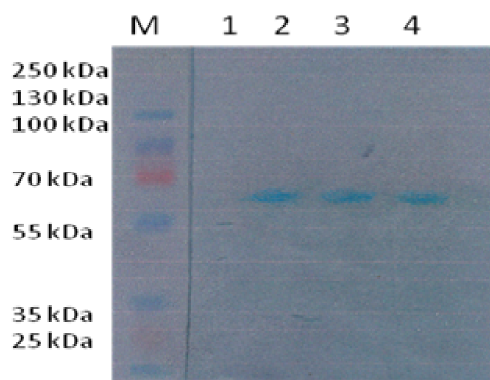

Fig. 3: Western blotting of specific anti-dengue IgY. Protein markers (M). IgY before immunization (Lane 1). IgY after immunization at 2 weeks (Lane 2); 4 weeks (Lane 3) and 6 weeks (Lane 4).

DISCUSSSION

In recent years, immunoglobulins obtained from avian egg yolks are increasingly finding favor to replace mammalian antibodies for diagnostic and therapeutic applications. The production of antibodies in chicken is less expensive, easy and non-invasive with high antibody yields (Fu *et al.,* 2006). In this study,We adopted a PEG extraction method followed by several steps of ammonium sulfate precipitation. The method is simple, efficient, and safe compared with water dilution, chloroform extraction, and gel-filtration chromatography and therefore, it is suited to large-scale isolation of IgY from egg yolk. The PEG used in this process, which is of low toxicity, is widely used in pharmaceutical production. After purifying of IgY from egg yolk, the IgY concentration in egg yolk determined by spectrophotometer (Biorad, USA) and Bradford method. IgY was successfully elicited by immunizing the hens with formalin-inactivated dengue antigen emulsified in Freund's adjuvant. The IgY concentration in egg yolk increased during the immunization period until week 6 where it began to increase dramatically at 2 weeks and it reached a plateau at 4 weeks after immunization. After week 6 the levels decreased gradually. These results indicated that chickens require about two weeks for antibody production and it also indicates that leghorn hens, as the host for the production of anti-dengue IgY, show the remarkable ability to rapidly and efficiently generate an abundant IgY and provide specific IgY in a noninvasive way. The injection of the antigen by the intramuscular route results in higher antibody levels by day 28 after immunization, and the resulting antibodies also exhibit higher specificity, being over 10 times more specific when compared with chickens immunized with the same antigen (Pauly *et al.,* 2009; Hirai *et al.,* 2010). Chickens, immunized by the intramuscular via, continue producing specific antibodies during more than 200 days (Sui *et al.,* 2011). Chickens can also tolerate the use of common immunological adjuvants, such as Freund's adjuvant.

AGPT are able to detect the presence or absence of antibodies to any virus. AGPT reactions between dengue antigen and IgY after immunization showed that IgY positive anti dengue if the precipitation lines from the IgY into the well. One line of precipitation was produced by IgY from 2; 4 and 6 week after immunization but not before immunization. The AGPT test was a satisfactory and uncomplicated technique for detecting precipitating antibodies against dengue virus. AGPT is simple and economical and diagnostic results are obtained sooner. It is a well-known concept that the immune response is more potent when the distance between the antigen source and the immune system increases. Therefore, to obtain immunoreagents containing antibody titers against mammalian antigens, chickens are better and cheaper than mammals (Lu *et al.,* 2009; Wang *et al.,* 2011).

The anti-dengue IgY produced by the immunized hens increased over time, as revealed by ELISA. The specific IgY titer increased starting the second week after the first immunization, with the titer peaking at the fourth week. After 6 weeks, the antibody titer slowly declined. Titer of anti-dengu IgY in yolk at

2; 4; 6 weeks after immunization demonstrated significantly higher than that of before immunization. It indicates that lohman hens, as the host for the production of anti-dengue IgY, show the remarkable ability to rapidly and efficiently generate an abundant IgY and provide specific IgY in a noninvasive way. Chickens, as a source of desired antibodies, represent an alternate animal system that offers some advantages with respect to animal care, high productivity and special suitability of avian antibodies for certain diagnostic purposes.

SDS-PAGE was applied to confirm isolation of immunoglobulin Y. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing two major bands of 66 and 26 kDa, and three minor bands of 145; 45 and 33 kDa. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing 66 kDa heavy chain and 26 kDa light chain. The general structure of the IgY molecule is the same as the IgG molecule with two heavy (H) chains and two light (L) chains but IgY has a molecular mass of 180 kDa which is larger than that of mammalian IgG (150 kDa). The molecular mass (67-70 kDa) of the H chain in IgY is larger than the H chain from mammals (50 kDa). The greater molecular mass of IgY is due to an increased number of heavy-chain constant domains and carbohydrate chains (Alexander *et al.,* 2009). IgG has 3 C regions (Cγ1–Cγ3), while IgY has 4 C regions (Cυ1-Cυ4) and the presence of one additional C region with its two corresponding carbohydrate chains logically results in a greater molecular mass of IgY compared with IgG.

The presence of anti-dengue IgY in egg yolks was confirmed by Western blotting. IgY obtained from hens at 2; 4; 6 weeks after immunization but not before immunization, showed the stronger bands with a single protein band of molecular weight protein (66 KDa). The results indicated the presence of specific anti dengue IgY. The immunization of chickens provides an attractive alternative to using mammals as hosts for antibody production. IgY is the major low molecular weight immunoglobulin in oviparous animals. This type of antibody has distinctive properties which can be exploited in various ways in research, diagnostics and therapy. One important advantage arises from the phylogenetic distance and genetic background that distinguishes birds from mammals. This improves the likelihood that an immune response will be elicited against antigens or epitopes that may be non-immunogenic in mammals. The deposition of IgY into the egg yolks of the immunized bird then provides an elegant source of polyclonal immunoglobulins. Since polyclonal IgY can be recovered from the eggs of laying hens for prolonged periods, this approach provides a longterm supply of substantial amounts of antibodies.

CONCLUSION

The results presented in this study indicate that immunization of hens with virus dengue could be a strategy to obtain at low cost a relatively high concentration of anti-dengue egg yolk IgY, could be an useful tool for research, diagnosis and therapy of dengue infection. Our results also suggest that egg yolk from immunized laying hens may offer a new large-scale source of low-cost antibodi.

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