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ORIGINAL ARTICLE

Development of a novel diagnostic kit candidate to detect dengue antibody, using IgY and protein a positive Staphylococcus aureus

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ABSTRACT

Early diagnosis of dengue hemorrhagic fever (DHF) is very important in determining further management. Diagnostic kits are intended as relatively faster, easier, and cheaper diagnostic tools. The objectives of this experimental laboratory study was to prepare a diagnostic kit candidate to detect dengue antibody in human serum using co-agglutination methods by utilizing protein A positive Staphylococcus aureus as a carrier to bind Fc immunoglobulin fractions without altering its ability to bind antigen. To enhance the sensitivity and specificity of the diagnostic kit, chicken IgY was used as one of the components. Rabbit immunoglobulin chicken anti-IgY was used as an intermediary connection between protein A positive and Staphylococcus aureus with chicken IgY. Solution A contains Staphylococcus aureus Cowan I and rabbit immunoglobulin chicken anti-IgY serum while Solution B contains anti-dengue chicken serum and dengue antigen. The laboratory experiments produced a formula of A: B = 1~3: 1~3 (v/v), which was then tested to human serum and compared with IgM and IgG ELISA tests as the gold standard. Data on the results of the test were statistically analyzed using chi-square test. Of the 65 qualified samples used, 47.69% resulted in true positive and 41.54% resulted in true negative. Meanwhile, the remaining samples demonstrated false negative (6.15%), positive predictive value (91.2%) and negative predictive value (87.1%). The sensitivity and specificity were 89% and 90%, respectively, with no significant differences ($p>0.05$). Therefore, co-agglutination method using S. aureus with protein A positive as a carrier can be proposed as a diagnostic kit candidate to detect human serum dengue antibody to screen dengue hemorrhagic fever cases.

Keyword: antibody, dengue, diagnostic kit, protein A, *Staphylococcus aureus*

INTRODUCTION

Dengue virus infection is currently regarded as the most important arboviral disease internationally, as over 50% of the world's population live in areas where they are at risk of the disease and approximately 50% live in dengue endemic countries[1]. The clinical manifestations of this infection range from asymptomatic infections to a severe disease characterized by hemorrhage and shock[2].

Early diagnosis of dengue hemorrhagic fever (DHF) is very important in determining further management. The case fatality rate (CFR) of patients diagnosed and treated late is 26%[3], whereas the CFR for early detection cases is less than 1%[2]. Several methods have been used for the serological detection of dengue virus-specific antibodies, including the hemagglutination inhibition (HI) test, the neutralization test, the indirect immunofluorescent antibody test, ELISA, complement fixation, dot blotting, Western blotting[4], and the rapid immunochromatography test (for which many commercial kits are available)[5].

Among these, capture IgM and/or IgG ELISA, antigen-coated indirect IgM, and/or IgG ELISA are the most commonly used serological techniques for the routine diagnosis of dengue virus infection due to their high specificity and sensitivity; however, they are technically difficult to do in facilities with limited resources and poor human resource skills, especially at a peripheral area in developing countries[6]. Recently, the detection of the NS1 antigen in the blood has become the most current and very popular diagnostic method for diagnosing this infection. This viral protein is secreted in the blood and can be detected by ELISA or immunochromatographic tests. A positive result of the test highly suggests a dengue case but a negative result does not rule out a dengue infection[7]. This study was an experiment to prepare a diagnostic kit candidate to detect dengue antibody with a high-level of sensitivity and specificity but relatively faster, easier, and cheaper than the gold standard. The diagnostic kit to be developed used the co-agglutination method using protein A positive *Staphylococcus aureus* as a carrier. This kit was to be compared with the ELISA as the gold standard.

METHODS AND SUBJECT

The diagnostic kit candidate consisted of solution A and solution B. Solution A was a mixture of S. aureus Cowan I intact in solution tris-HCl buffer with rabbit anti-chicken IgY serum. The solution A mixture was made in various proportions (v/v) and each mixture was shaken until it mixed well and then incubated for at least 60 minutes at room temperature. The resulting solution was then kept in a sealed tube at a temperature of 40^{o}C. Solution B was a mixture of dengue virus antigen with chicken anti-dengue serum. Serum was incubated in 56 ^{o}C water bath for 30 minutes and serial dilutions with dilution factor 2 between solution B and physiological saline was performed. The mixture was shaken until homogeneous and then incubated for at least 60 minutes at room temperature. The resulting solution was then kept in a sealed tube at 40^{o}C[8].

Diagnostic kit candidate component preparation

Preparation of rabbit anti-chicken IgY serum

To get the rabbit anti-chicken IgY serum, researchers immunized rabbits with chicken IgY. One rabbit was immunized, while the other one served as a control. Chicken IgY dose of 1 ml (1 mg/ml) were injected intravenously. The injection was performed three times a week for four (4) weeks. One week after the last injection, the serum was harvested intravenously and the serum titers of rabbit anti-chicken IgY was measured by the AGPT (agar gel precipitation test) method[9,10].

Preparation of chicken IgY Anti-Dengue

We used only one type of dengue antigen because there are cross-reactions between different dengue serotypes[11]. In this study, type 3 dengue antigen was used because it is a dominant serotype in Indonesia and often caused severe dengue cases[12,13]. Before immunization, the chicken serum was checked first using the hemagglutination (HI). If the result was negative, the immunization was then performed on two specific-pathogenic-free (SPF) 8-week old laying hens weighed 1.5 kg. One different chicken

was used as a control. The titer of antigen used was 1/51 hemagglutination assay (HA) unit. The hen was immunized subcutaneously with dengue antigen type 3 to generate IgY of anti-dengue type 3. Booster injections conducted at week 0, 2,3, and 4 to reach a high titer. For the first injection, the antibody was emulsified in complete Freund's adjuvant (CFA) with a total volume of 1 ml and incomplete adjuvant was used for the three subsequent boosters. The titer would be high at day 35 and was expected to persist in 1-6 months.[14]

Optimization of Diagnostic Kit candidate (Solution A and Solution B)

Optimization was conducted on Solution A and solution B using the following principles: the mixing between Solution A and Solution B may not cause co-agglutination. Formulation of Solution AB would be used as the diagnostic kit candidate when the positive control produced coagglutination and the negative control did not produced co-agglutination.

Testing on samples of diagnostic kit candidate

For testing, 25-50 µl diagnostic kit candidate was placed on a preparation slide by dropping it on the glass, followed by a drop of sample (25-50 µl) placed next to the first drop. The diagnostic kit and sample drops were then mixed the tip of the pipette. The coagglutination was examined within 5-10 minutes.

ELISA sample

One hundred samples were collected randomly from patients with dengue hemorrhagic fever that had been tested by ELISA at Pramita laboratory. The criteria for sample to be used in this study were samples that had been examined using the ELISA dengue IgM and IgG. A positive ELISA test result was defined as an IgM or IgG titer of > 11 PBU (Pan Bio Unit) and the negative result was defined as having <9 PBU. An IgM or IgG titer between 9-11 PBU was considered to be a doubtful result. Samples with a dubious result or that were tested for IgM and IgG only were not used in the study.[15]

Data analysis

Statistical analysis was performed using the chi-square method and the sensitivity, specificity, efficiency, positive predictive value (PPV), and negative predictive value for the assay were calculated.[16]

RESULTS AND DISCUSSION

Solution A is a mixture of S. aureus Cowan I intact in solution tris-HCl buffer with rabbit anti-chicken IgY serum. Rabbit anti-chicken IgY serum titer was measured using the AGPT method. Middle wells were filled with pure chicken IgY, while the sixth well outside was filled with rabbit serum with a dilution factor of ½. A positive reaction was indicated by the formation of precipitation line between the outer wells and middle well. The measurement results of serum titers of rabbit anti-chicken IgY was 1/8.

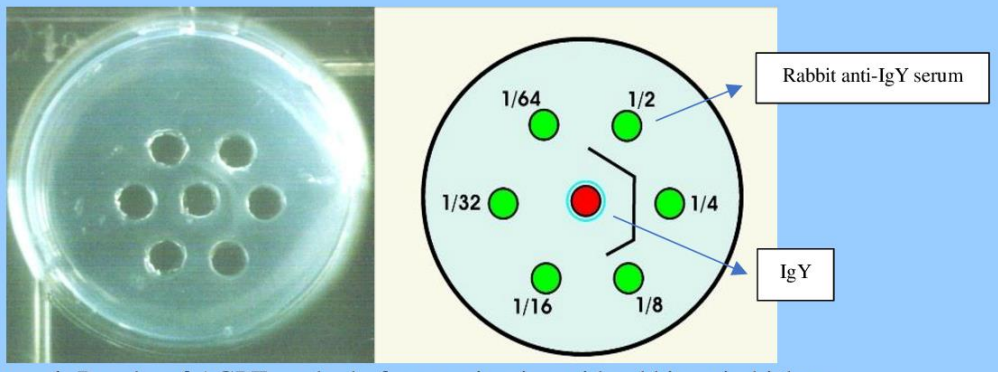

Figure 1. Results of AGPT method of serum titration with rabbit anti-chicken Ig Y. The titer of each are 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 (clockwise).

Solution B was a mixture of dengue virus antigen type 3 with chicken anti-dengue type 3 serum**.** Virus antigen type 3 was obtained from PT Bio Farma, while the chicken anti-dengue serum was created by injecting a dengue antigen to chicken. To harvest the chicken serum, as many as 15 cc of blood was drawn from each chicken. The chicken serum was then checked using the HI method at the virology laboratory of PT. Bio Farma. The average antibody titer which obtained was 1/80 HA unit. Based on the box titration using HI method, it was revealed that the optimal chicken serum titers for the anti-dengue type 3 was 1/20 HA Unit and 1/40 HA unit for type 3 dengue antigen titer.

Table 1. Box titration of dengue antigen type 3 and chicken serum anti-dengue type 3

	chicken serum dengue antigen type 3			
Anti-dengue type 3	1/10	1/20	1/40	1/80
1/10	+	+	+	+
1/20	+	+	+	-
1/40	+	+	-	-
1/80	+		-	-

After all components were complete, then optimization was performed. Solution A consisted a mixture of a suspension of S. aureus Cowan 1 strain with rabbit anti-IgY serum. The optimal composition for this solution was 4 ~ 6 : 2 ~ 4 (v/v). To avoid false positive reactions or to improve specificity, saturation by adding normal rabbit IgG was conducted. This was useful to prevent the bond between negative serum samples (IgG in human serum) and protein A which can cause coagglutination. The composition of Solution A : normal rabbit IgG was 1 ~ 3: 2 ~ 4 (v/v). Solution B consisted of type 3 dengue virus antigen titer 1/40 and a chicken anti-dengue serum titer of 1/20. The titer was obtained by box titration using the HI method (Table 2). The optimal composition between dengue virus antigen with anti-dengue chicken IgY in this solution was 1 ~ 3: 1 ~ 3 (v/v). The diagnostic kit candidate was made by mixing Solution A and Solution B into Solution AB. The optimal composition of Solution AB as the diagnostic kit candidate was A: B = 1 ~ 3: 1 ~ 3 (v/v). The Solution AB did not cause coagglutination.

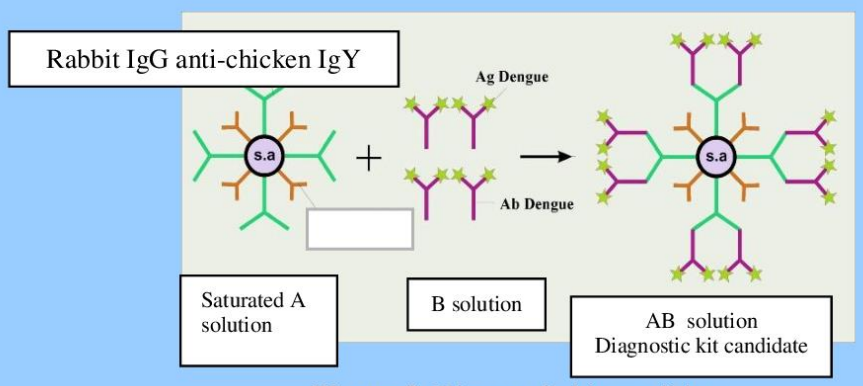

Figure 2. Diagnostic kit candidate

The diagnostic kit candidate was then tested in human serum. The expected results were results of tests on the positive control, i.e., serum that contains dengue antibodies, that showed positive coagglutination, whereas the negative control, i.e., serum containing no dengue antibodies, did not show coagglutination.

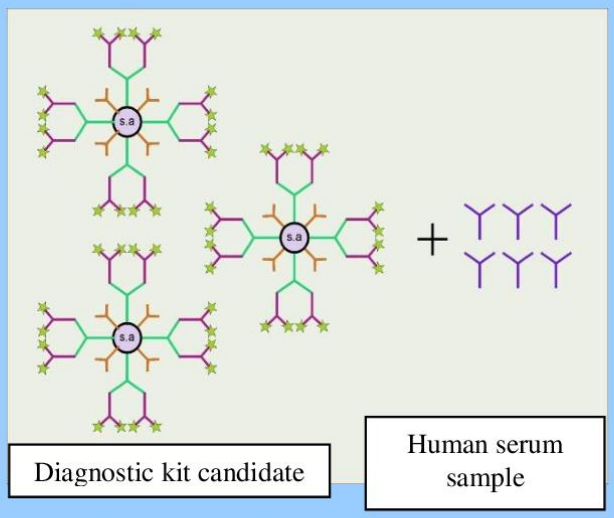

Figure 3. Diagnostic Kit candidate testing on samples

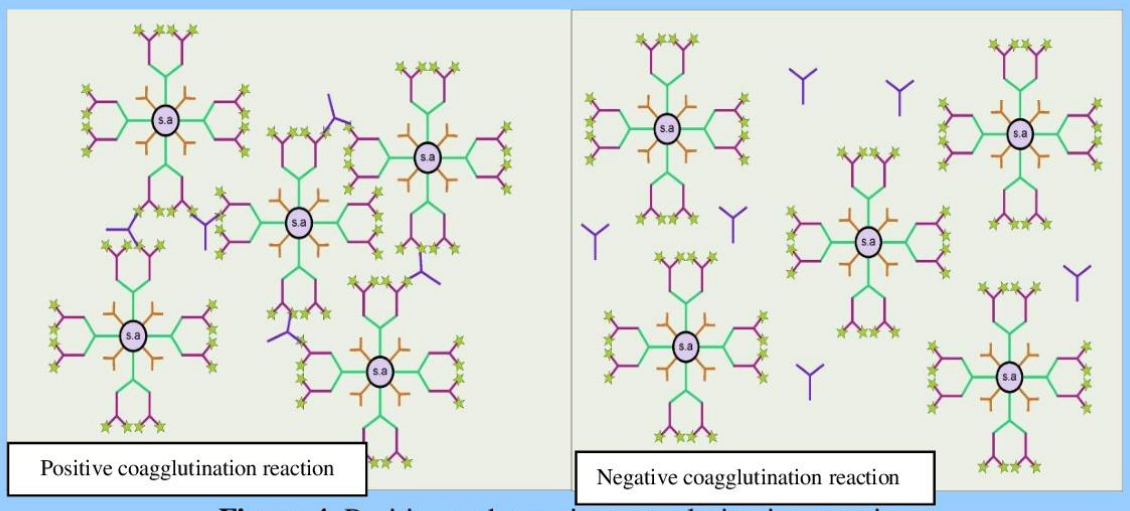

Figure 4. Positive and negative coagglutination reaction

Visually positive test results looked like fine white granules at the bottom of the droplets, surrounded by a clear colorless liquid. Meanwhile, the negative test results would present as cloudy suspension. Optimum readings can done within 5-10 minutes.

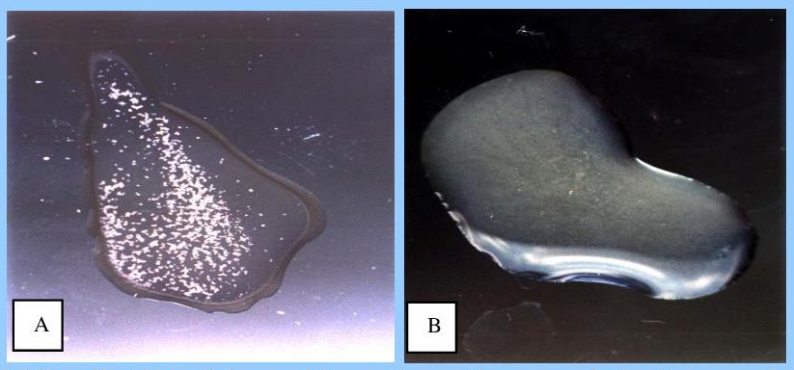

Figure 5. Visualization of the positive and negative coagglutination reactions result (A) positive coagglutination and (B) negative coagglutination

An appropriate antigen and antibody titer is required to form an optimal coagglutination. According to Paul WD (2008), a phenomenon that occurs in a precipitation reaction also applies to the coagglutination reaction. Therefore, the coagglutination reaction can also apply to the "Marrack or Lattice theory." The theory describes the maximum precipitate in an equilibrium zone. Based on this theory, in order to precipitate, the proportion between the antigen and antibody has to be precise. Precipitation will not occur in a reaction containing excess antibody (prozone effect) or less antibody (postzone effect)[17]. Thus, the composition of the A: B = 1 ~ 3: 1 ~ 3 (v/v) preparations with each titer have been optimized and is expected to produce an optimal coagglutination. To determine the sensitivity and specificity levels, the results of the diagnostic kit candidate examinations were compared to the results of the ELISA examinations.

Of the 100 samples received, only 65 samples qualified. The samples were tested with PANBIO dengue IgM ELISA (E-DEN01M) and PANBIO Dengue IgG ELISA (E-DEN01G) to determine the IgM and IgG titers. This was then followed by a test using the optimized AB formulation with coagglutination method.

Table 2. Results of sample testing using coagglutination method as compared to ELISA

Parameter	Result of examination (n=65)
True positive	31 (47.69%)
False positive	3 (4.61%)
True negative	27 (41.54%)
False negative	4 (6.15%)
Sensitivity	89%
Specificity	90%
Positive predictive value	91,2%
Negative predictive value	87,1%

From the test results of the 65 samples that were compared with the ELISA, the following results were obtained: 31 true positive samples (47.69%), 3 false positive samples (4.61%), 27 true negative samples (41.54%), and 4 false negative (6.15%). The sensitivity levels of the PANBIO dengue IgM ELISA (E-DEN01M) were 94.7% for primary infection and 55.7% for secondary infection while the sensitivity levels of the PANBIO Dengue IgG ELISA (E-DEN01G) were 91.4% for

primary infection and 96.3% for secondary infection [15]. The ELISA sensitivity is high, mainly determined by the properties of enzymes that can amplify the substrate change, while the sensitivity of the coagglutination method is determined by the more easily formed lattice formation that is determined by the growing particles formed and its ability for multivalent binding.[17]

The sensitivity and specificity levels obtained for the diagnostic kit candidate were 89% and 90%, respectively. The terms of sensitivity and specificity are usually used to describe the accuracy of laboratory test results. The sensitivity of a test describes the ability of a diagnostic test to detect cases of disease or the possibility of getting a positive test result on a group of subjects who are sick. Meanwhile, the specificity describes the ability of a diagnostic test to exclude cases that are not a disease or the possibility of getting a negative result on a group of healthy subjects[18]. Differences in sensitivity and specificity values between the coagglutination method and ELISA method in this study were not statistically significant.

Table 3. Chi-square test result for comparison of sensitivity and specificity between coagglutination method compared and ELISA method.

Variable	λ^2	P-value
Sensitivity	0,0145	p>0,05
Specificity	0,0164	p>0,05

The use of a chicken IgY is very significant in improving sensitivity. According to the European Center for the Validation of Alternative Method in 2000, the protein A cannot bind to chicken IgY that necessary to use the indirect method to make it happen. As the bridge linking, rabbit IgG anti-chicken IgY is used [14]. The indirect method will further extend the circuit so that the structure design of the coagglutination method from the inside out will be as follows: S. aureus protein A positive, rabbit IgG anti-IgY chicken, chicken immunoglobulin anti-dengue, and dengue antigen.

The length of this circuit will further aggravate and increase the size of particles that will increase the sensitivity. The specificity of PANBIO dengue IgM ELISA (E-DEN01G) and PANBIO Dengue IgG ELISA (E-DEN01M) is 100% for both[14]. The high specificity of ELISA is determined by the type of antibody used. The ELISA is a method that uses a monoclonal antibody with a high specificity when compared to the polyclonal antibodies. Monoclonal antibodies are more specific than polyclonal antibodies since they are homogeneous antibodies produced by the plasma cell clone and have identical immunochemical properties. In addition, they also react to specific epitopes on the antigen.[19]

The specificity of coagglutination method is greatly influenced by the use of a chicken IgY. According to a previous study, the chicken IgY has advantages when compared to IgG of mammals, such as its capability to not react to the Fc receptor of mammals and rheumatoid factor and does not activate the complement system[8]. This is expected to minimize the possibility of false positives. Moreover, the saturation of protein A greatly affects the level of specificity. Protein A must be in saturated conditions binds to rabbit immunoglobulin to prevent it from binding to human IgG from negative serum samples, which will cause false positives.[20]

Based on table 3 and 4. the sensitivity and specificity levels of the coagglutination method is quite high and are not significantly different from those of ELISA. Besides having a high sensitivity and specificity, this method has other advantages, such as (1) the process is relatively easy, just involving mixing Solution A and B and sample preparation which does not require special skills, (2) only require simple tools, such as preparation slides, pipettes, and tools for sample preparation which can easily be accessed in laboratories with limited resources, and (3) only requires one serum sample and results can be obtained quickly, with optimum results can be obtained within 5-10 minutes.

Based on the results of this study, it is possible to develop this diagnostic kit candidate to diagnose dengue in health facility with limited resources and for screening purposes.

CONCLUSION

There is no significant difference in the sensitivity and specificity levels (p>0,05)) of the co-agglutination method when compared with the ELISA method. Thus, the co-agglutination method using *S. aureus* with protein A positive as a carrier can be proposed as a diagnostic kit candidate to detect human serum dengue antibody to screen dengue hemorrhagic fever cases.

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DECLARATION OF INTERESTS

There is no conflict of interest in this study

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