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Research Report

## AN EVALUATION STUDY OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) USING RECOMBINANT PROTEIN GRA1 FOR DETECTION OF IgG ANTIBODIES AGAINST TOXOPLASMA GONDII INFECTIONS

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### ABSTRACT

*Toxoplasmosis is an infectious disease caused by *Toxoplasma gondii*, an intracellular protozoan parasite that live inside the cells of the reticulo-endothelial and parenchymal cells of human and animals (mammals and birds). Some cases of toxoplasmosis usually have no symptoms, but in any cases caused severe symptoms, such as hydrocephalus, microcephalus, intracranial calcification, retinal damage, brain abscess, mental retardation, lymphadenopathy, and others. Its severe symptoms usually showed a long time after first exposure, except symptoms showed by congenital transmission caused by infected mother. Early diagnosis is important to prevent the illness but methods for toxoplasmosis screening are still too expensive for developing country. Enzyme-linked immunosorbent assay (ELISA) allow the testing of a large number samples within short time frame and based on antibody or antigen detection. This study aimed to know the sensitivity and specificity of recombinant protein GRA1 as antigen using ELISA methods. We tested the sensitivity and specificity of GRA1 protein as antigen in ELISA methods to diagnose toxoplasmosis and compared with ELISA Kit Commercial. Reliable laboratory testing is important to detect *Toxoplasma gondii* infection, and focused to improving the low cost and easy-to-use diagnostic instrument. Seventy sera collected and tested using both indirect ELISA, commercial ELISA kit and GRA1 protein coated as antigen. Fourty eight and fifty one samples showed positive IgG antibody result of ELISA-GRA1 and ELISA kit. Negative sample tested by ELISA-GRA1 was 22 samples and 19 sample tested by ELISA Kit. The sensitivity and specificity of GRA1-based on ELISA were 100% and 86.36%, positive prediction value (ppv) was 94.11%. These data indicate that the recombinant protein GRA1 is a highly immunogenic protein in human toxoplasmosis and become a promising marker for the screening of toxoplasmosis.*

**Keywords:** *Toxoplasmosis, recombinant protein GRA1, ELISA, Sensitivity, Specificity*

### ABSTRAK

*Toksoplasmosis merupakan penyakit menular yang disebabkan oleh protozoa parasit intrasel spesies *Toxoplasma gondii*, yang hidup di dalam sel endotelial dan perenkim manusia dan hewan (mamalia dan aves). Sebagian besar kasus toksoplasmosis tidak menunjukkan gejala, namun pada beberapa kasus menyebabkan kondisi yang kronis seperti hidrosefalus, mikrosefalus, kalsifikasi intrakranial, kerusakan retina, abses otak, keterbelakangan mental, limfadenopati, dan gejala lainnya. Gejala tersebut akan tampak beberapa waktu setelah infeksi pertama, kecuali pada kasus kongenital toksoplasmosis dimana bayi terlahir dalam kondisi cacat akibat infeksi pada ibu pada trisemester kedua. Diagnosis toksoplasmosis merupakan kunci utama dalam pencegahan munculnya gejala namun prosedur diagnosis masih tergolong cukup mahal di Negara Berkembang. Salah satu metode diagnosis toksoplasmosis adalah dengan metode Enzyme-Linked Immunosorbent Assay (ELISA) yang dapat melakukan pemeriksaan untuk sampel dengan jumlah besar dalam waktu singkat berdasarkan reaksi antigen dan antibodi. Bentuk upaya pengembangan instrumen diagnosis ELISA adalah penggunaan protein rekombinan sebagai antigen, seperti protein GRA1. Penelitian ini bertujuan untuk mengetahui nilai sensitivitas dan spesifitas protein rekombinan GRA1 sebagai antigen menggunakan metode ELISA. Uji sensitivitas dan spesifitas protein GRA1 sebagai antigen dilakukan dengan membandingkan dengan ELISA Kit yang telah dijual bebas pada 70 sampel. Sebanyak 48 dan 51 sampel serum menunjukkan*

hasil positif toksoplasmosis yang diuji menggunakan ELISA-GRA1 dan ELISA Kit. Sebanyak 22 sampel menunjukkan hasil negatif toksoplasmosis menggunakan metode ELISA-GRA1, sedangkan 19 sampel negatif menggunakan ELISA Kit. Nilai sensitivitas GRA1 sebagai antigen adalah 100%, spesifitas sebesar 86,36%, dan nilai prediksi positif sebesar 94,11%. Berdasarkan data tersebut, protein rekombinan GRA1 diketahui memiliki aktivitas immunogenik yang tinggi pada penderita toksoplasmosis dan dapat digunakan sebagai penanda untuk diagnosis toksoplasmosis sehingga pengembangan alat diagnosis dengan biaya rendah dapat dilakukan.

**Kata kunci:** Toksoplasmosis, Protein Rekombinan GRA1, metode ELISA, Sensitivitas, Spesifitas

## INTRODUCTION

Toxoplasmosis is disease caused by infection of obligate protozoan parasite, called *Toxoplasma gondii*. Human can be infected by *T. gondii* in many ways such as, congenital, consumption habits (raw meat, raw vegetables), activity with soil/meat without protection, blood transfusion, organ transplantation, and etc. Oocyst become infective stage when passed out from definitive host and contaminated with water sources, soil, and plants. Most of toxoplasmosis are asymptomatic but can be serious problems in immunocompromised patients and newborns with congenital toxoplasmosis. Infection can cause encephalitis in immunocompromised hosts, chorioretinitis in immunocompetent hosts or serious congenital disease in developing fetus if pregnant women become infected for the first time during pregnancy.<sup>1</sup> More than 60% world population are toxoplasmosis, and 90% of it asymptomatic even they have Tg antibodies. It depends on the individual immune responses to prevent the symptoms.<sup>2</sup>

Detection for toxoplasmosis usually using serological methods, such as Dye Test (DT), Modified Agglutination Test (MAT), Enzyme-Linked Immunosorbent Assays (ELISA), immunosorbent agglutination assay (ISAGA), Indirect Fluorescent Antibody Test (IFAT) and Indirect Haemagglutination Assays (IHA) to detect Tg antibody. Demonstrating the parasite in tissue can be done by culture of parasite (in vivo and in vitro) and detection of specific nucleic acid using DNA probe, PCR and L-AMP methods. Enzyme-Linked Immunosorbent Assays (ELISA) is a popular and commercially available easier methods for clinical detection of toxoplasmosis. Commercial ELISA kits use antigen from native tachyzoites which grown in mice or tissue culture and probably contain varying amounts of extraparasitic material.<sup>3</sup> Limitations of the tachyzoite antigen for serologic tests can be serious problems, another antigens should become an alternative test, such as using purified recombinant antigens which expressed by tachyzoites and bradyzoites. However, the whole tachyzoite native antigen test is difficult to standardize and in some cases produce false positive reactions.<sup>4</sup> Tachyzoites is not the only component which could activated the immune response to produce antibody, expression of excreted-secreted antigen from bradyzoites can induces antibody production and IgG specific Tg always exist in infected host lifetime.

GRA1 has been identified as excreted-secreted antigen (ESA) in tachyzoites and crossreactive with bradyzoites.<sup>5</sup> It located in dense granule of both tachyzoites and bradyzoites, and used as a marker of secretory organelles of *Tg*. It always secreted in lumen and potentially can be identify in body fluid of infected host. GRA1 induces humoral and cellular immune responses in the chronic phase of the infection in mice and humans and increasing production of antibody and IFN- $\gamma$ . GRA1 epitopes present in MHC class I molecules during infection and induces specific CTLs.<sup>6-8</sup> GRA1 was secreted into the lumen of the parasitophorous vacuola as a soluble protein and associated with the membranous tubular network peripherally.<sup>9</sup> GRA1 needed for secretion of 3 secretory organelles of *Tg* and became marker of dense granule proteins.<sup>10</sup> Vaccination using GRA1 protein show the activity of CD8<sup>+</sup> T-cells against parasite-infected cells and a GRA1-transfected cell line.<sup>7</sup>

The costs for serologic testing in developed countries are not prohibitively high. However, in developing countries there is alternative low-cost test with the same sensitivity and specificity. The costs for the development of instrument depend on the efficient production of recombinant antigens.<sup>11</sup> Previous study, in same project, have developed an efficient system for the production and purification of GRA1 proteins and have been tested for immunogenic activity. Based on the ability of GRA1 to stimulated immune response, we tried its ability as antigens to develop the diagnostic tools. Sensitivity and specificity of GRA1 as antigens in ELISA methods will compared with commercial ELISA kit to detect Tg-IgG antibodies.

## MATERIAL AND METHOD

Seventy human sera were obtained from previous study in central java population and approved by ethical committee of Faculty of Medicine Universitas Gadjah Mada for research in human subject.<sup>12</sup> Sera were tested using ELISA methods and separated for both ELISA kit test and ELISA-GRA1 coated protein as antigen. Preparation of recombinant protein GRA1 consist of isolation, characterization, cloning, expression, and purification of GRA1 protein. Isolation, characterization, cloning, and expression of GRA1 protein were worked by previous researcher in same project<sup>13,14</sup> and culture of *E.coli* inserted GRA1 protein were stored in 4°C until we

used to this study. We were re-culture the recombinant *E. coli* and isolated GRA1 protein used sonication to break the bacterial membrane. GRA1 protein purified using Ni-ted Profino Coloumn Chromatography and electrophoresis to confirmed the result.

Protein recombinant GRA1 was diluted to the optimized concentration of 2 µl in 200 µl Biorad Protein Assay (PBA) and 798 µl H<sub>2</sub>O then optical density checked by using spectrophotometer with wavelength 595 nm. GRA1 protein level measured following this formula; (Optical Density+0.057)/0.0465. Optical density was found 0.02885 and counted using that formula showed GRA1 protein level was 0.5602 µg/µl. Counting of protein level was needed to calculated the volume of the protein which is incubated as antigen and must reach 5 µg/100 µl. Each microwell was added by 50 µl of protein solution and coated overnight at 37°C using coating buffer (Na<sub>2</sub>CO<sub>3</sub> and NaHCO<sub>3</sub>). The processes were done step by step, consist of blocking process, samples additions, conjugates (antihuman IgG alkaline phosphatase), substrate (pNPP), and stop solution. Washing solution was added three times after each processes to removed all unbounded particles. Blocking buffer (PBS-BSA 1% pH 7.00) was added and incubated at 37°C in hour. Human sera and antihuman IgG alkaline phosphatase (conjugate) were diluted 10 times and 5000 times, respectively, and incubated. P-nitrophenyl phosphate as substrate was diluted in substrate buffer 1mg/ml (Diethanolamin and MgCl<sub>2</sub>) and 150 µl was added into each well then incubated 30 minutes. Reaction was stopped immediatelly with addition of stop solution containing HCl. The quantitative result was measured by ELISA reader to detect optical density (OD). Cut off value was counted by mean of negative control OD.

Effectiveness of GRA1 as promising-antigen was evaluated by ELISA kit commercial (GenWay BioTech) coated with native tachyzoites. Procedure of ELISA kit which used were followed the manufacture instruction. The kit consist of dilution buffer, washing buffer, negative and positive control, 4 type of calibrator to differentiate negative, low and high positive antibodies concentration, and stop solution.

## RESULT AND DISCUSSION

Serodiagnostic using recombinant proteins of Tg evaluated by indirect ELISA and compared with commercial ELISA kit. Sensitivity and specificity were measured to

detect the effectiveness of GRA1 proteins as antigens. As shown in Table 1, there were 51 positive and 19 negative samples by ELISA-GRA1, while there were 48 positive and 22 negative samples tested by ELISA kit.

Commercial ELISA kit usually using native tachyzoites antigens coated in microplate and distributed worldwide to diagnosis of *Toxoplasma gondii* infections. This tools is a high-cost instrument among laboratories and not always accurate because often produces false positive reactions.<sup>4</sup> While toxoplasmosis diagnostic is an important test for human in every social-economic status, developing a low-cost diagnostic tools really important to support the health status and epidemiological screening of infected disease in populations.

The results of this study explicitly show that GRA1 antigen is suitable for detecting serum antibody to Tg infections and clearly distinguished mean of Optical Density (OD) and 95% of Confidence Interval (CI), the method is able to differentiate seropositive and seronegative of Tg-IgG sera. There were 51 positive and 19 negative samples tested by ELISA-GRA1, while 48 positive and 22 negative samples tested by ELISA kit (GenWay BioTech). All observed sensitivity and specificity estimates greater than 80%. Sensitivity of GRA1 is 100% and specificity reach 86.36%. Based on a sensitivity and specificity of 80%, the observed sample size was sufficient to estimate good sensitivity and specificity as diagnostic tools.

Dense granule proteins function is to manage modification of the parasitophorus vacuola and intake nutrition from cytoplasm of infected cell.<sup>5,15-18</sup> This protein was needed by tachyzoites for continuing their development in infected cell and replicate inside of parasitophorous vacuolar membrane.<sup>18</sup> Most of dense granules proteins secreted in parasitophorous vacuoles and increase following the number of parasite infections. A molecule became potential antigen if it have weigh over 1 kD, complex structure, and a stabil molecules.<sup>19</sup> GRA1 proteins have immunogenic and antigenic activity.<sup>6,7,20</sup> Vercammen *et al.* (2000) was reported the result of GRA1 vaccination induce humoral immune response in mice and produce IgG antibodies. Naturally, GRA1 induces secretion of IgG antibodies spesific to GRA1 and could be detected using serologic assay.

However, there is a significant advantage in the preparation of recombinant proteins over the preparation of crude Tg proteins. Recombinant Tg proteins could be produced economically and in large quantities by *E. coli* culture, but crude Tg antigens must be extracted from Tg

**Table 1.** Sensitivity and specificity of GRA1 recombinant protein as antigens for toxoplasmosis detections.

No. ELISA kit No. ELISA-GRA1	Positive of Tg IgG antibody	Negative of Tg IgG antibody	Total
Positive of Tg IgG antibody	48	3	51
Negative of Tg IgG antibody	0	19	19
Total	48	22	70

within the animal model. These crude extracts contain large amounts of proteins and other macromolecules, and most of them can influence the results of the test.<sup>21</sup>

Purified recombinant proteins could be an alternative substances to detect serum antibodies and will allow better standardization of the immunoassays.<sup>21–23</sup> Furthermore, a combination of recombinant antigens may enhance the sensitivity of an antibody-based assay. Several previous studies have found that recombinant antigens improve the serological diagnosis of *Tg* infection.<sup>22,24–26</sup> Moreover, recombinant antigens have the potential to be used in the creation of new instrument that differentiate recently acquired infections from those acquired in the more distant past.

## CONCLUSION

Our study showed high sensitivity and specificity of GRA1 recombinant protein as antigens for detections of toxoplasmosis using Enzyme-Linked Immunosorbent Assays. GRA1 recombinant proteins became promising antigens performed accurate result and should be develop as alternative tools for *Tg* antibodies detection in toxoplasmosis suspect.

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