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Original Article

Identification of *Toxoplasma gondii* Excretory-Secretory Antigens from Propagation in Mice as Toxoplasmosis Vaccine Candidate

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Abstract

The aim of this research is to get some ESAs of *T. gondii* tachyzoite from propagation in mice and ESAs having the character of immunogenic as candidate of toxoplasmosis vaccine. The long-range target of this research is get subunit vaccine of toxoplasmosis. The methods used cover: propagation of *T. gondii* in mice and cropping ESAs at intraperitoneal fluid and fractination ESAs by SDS-PAGE, antibody making to ESAs, and determination antigenicity by western blot. The results of research founded 13 ESAs: 125.5; 100.9; 70.2; 56.7; 45.6; 40.4; 35.3; 34.1; 29.5; 27.4; 24.9; 20.7 and 19.7 kDa. ESAs having the character of antigenic were molecule weighing 100.9; 56.7; 35.3; 34.1; 24.9; 20.7 and 19.7 kDa, and 100.9; 56.7; 24.9; 20.7 and 19.7 kDa represent major antigenic.

Keywords: *Toxoplasma gondii,* excretory-secretory antigens, vaccine.

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Introduction

The purpose of this study is to obtain excretory-secretory antigens (ESAs) that were antigenic propagation results that can be developed for toxoplasmosis vaccine. Toxoplasmosis is a disease that can attack the birds and mammalian including humans. In humans, the manifestation of the infection caused by *T. gondii* are defects in children born. One attempt to control toxoplasmosis among others by means of vaccines so that the host is able to defend against infections. Some techniques to get the vaccine for the prevention of disease already done but not getting the most. Some researchers have tried to use DNA vaccines to prevent infection with *T. gondii* in mice and using multiple antigens such as surface membrane antigen (SAG1) (Angus *et al.*, 2000), excretory-secretory of dense granule (GRA1) (Scorza *et al.*, 2003), GRA7 (Vercammen *et al.*, 2000), GRA4 (Desolme, *et al.*, 2000), ROP2 (Vercammen *et al.*, 2000; Leyva and Saavedra, 2001) and ROP1 (Chen *et al.*, 2001). The results showed that different protection against toxoplasmosis in mice. Among the existing vaccine candidates, MIC3 micronema protein (90 kDa) that was part of ESAs indicates a high potential due to its inherent nature strongly on host cells either on tachyzoite stage, bradyzoite and sporozoite of *T. gondii* (Garcia-Reguet *et al.*, 2000; Cerede *et al.*, 2002). Constraint is the use of sub-unit vaccine protein source used. Now this, the protein obtained by in vitro with breaking tachyzoite of *T. gondii* which requires large amount of charge produced little protein. On the other hand, tachyzoite of *T. gondii* can be propagated in the intraperitoneal cavity of mice in a relatively short time. *Toxoplasma gondii* in the intraperitoneal cavity will evolve and be excreted-secreted several proteins required for parasite development known as excretory-secretory antigen (ESAs). The proteins produced by the organelle rhoptry, micronema and dense granule (dense granule) and the protein can partially raise the host immune system in response to infection. Excretory-secretory antigens were obtained from propagation (in vivo) has protein conformation and function better than the ESAs protein isolated *T. gondii* by in vitro method. Some proteins can stimulate the emergence ESAs immunity in the infected host, so that the protein can be used as a sub-unit vaccine candidate.

To get ESAs *T. gondii* in vivo was done by propagation in the intraperitoneal cavity of mice, fractionated by SDS-PAGE and injection in experimental animals as well as to determine antigenicity with wertern blot.

Materials and Methods

The study used tachyzoite of RH strain *T. gondii*, Balb/C strain mice for propagation and rabbit for harvesting of antibody. A total of 1.10^7 tachyzoites of *T. gondii* injected in mice intraperitoneally. Mice were maintained 48 to 72 hours, and after showing sick, mice were sacrificed and the peritoneal cavity was washed with 5 ml PBS so tachyzoite can be collected. Liquid of washing results then centrifuged at 4000 rpm for 10 minutes. Supernatant was taken and put in a tube and ready for ESAs protein isolation (mufasirin, 1999).

Results of propagation supernatant was added ammonium sulphate saturated with 1: 1 ratio, mixed and incubated in 4°C overnight, then centrifuged at 1000 rpm for 1 h. Supernatant was discarded and the pellet was washed with PBS and centrifuged at 10,000 rpm for 2 hours. Washing is done 3 times in the same way. Pellet was dissolved in PBS and protein levels were measured with a spectrophotometer (mufasirin, 1999).

ESA fractionation performed by SDS-PAGE. Running gel was made and put in a glass plate, after stacking gel hardens put the prepared. Stackling running gel composition and gel made by mixing acrylamide, tris, 0.8% SDS, temed, APS and distilled water in a glass Beaker. Ten micrograms of protein sample was added laemly buffer with a ratio of 2: 1. It was boiling at 1000C for 5 minutes, then put into a mold column lies in the stacking gel and was running in the chamber filled with electrode buffer at 100 volt, 40 mA. As the standard marker used prestained protein molecular weight marker (Fermentas, Lot. 00.024.549). After running, the gel placed in the wash solution consisting of 25 ml of methanol, 3 ml acetic acid, and distilled water ad 100 ml for 30 minutes.

Performed repeated washing with a solution of the same composition with reduced ethanol and acetic acid addition of half of the previous 30 minutes. Further washing with a solution of 10% glutaraldehyde for 30 minutes and distilled. After washing the gel stained with AgNO3 for 15 minutes, then do the washing with distilled water twice each for 2 minutes. Given color development solution consisting of 3.7% formaldehyde, 5% zitronsauce and distilled water. After the protein bands visible then the reaction was stopped by adding 10% acetic acid. Results of gel that has seemed protein bands stored in 10% glycerol solution and ready to be documented. Calculation is done by comparing the molecular weight standard (mufasirin, 1999).

Immunization in experimental animals performed to obtain serum that contains antibodies against ESA of *T. gondii*. Healthy adult male rabbits were injected with 50-100 µg ESA of *T. gondii* previously added complete adjuvant (Sigma) with ratio 1:1. Subcutaneous injections made at four locations on the body that have loose skin. Injection was repeated with the same protein with the addition of incomplete adjuvant (Sigma) at 2 weeks after the first injection. Booster using the same protein with incomplete adjuvant in the same way. Booster performed every 2 weeks until a high antibody titer obtained. Before the first injection, was taking serum as a control. Measurement of antibody titers by Enzyme Linked Immunosorbent Assay (mufasirin, 1999).

Antigenic ESAs of *T. gondii* were identify by using western blot. Results of SDS-PAGE transferred to niroselulose membrane, further bloked with 1% BSA, overnight, at 4°C and continued washing with 0.05% tween 100 in TBS for 10 minutes and wash repeated 3 times. Membrane was placed in a solution of antibodies against ESA in PBS (1:50) and incubated 1 hour at room temperature with agitation and washed with 0.05% Tween 100 in TBS for 10 minutes. The washing was repeated 4 times in the same way. The membrane was incubated in a solution of conjugate (1:3000) (Santa Cruz, USA) for 1 hour at room temperature with agitation, followed by washing five times with 0.05% tween in TBS and 1 times without tween. Membrane was stained with Western Blue Substrate Ready. The reaction was stopped when it was visible protein bands by adding distilled water. Membrane was dried on Whatman paper and ready to be documented. Molecular weight calculation is done by comparing the antigenic protein bands were visible with standard marker stained with silver nitrate (mufasirin, 1999).

Result

Results of ESAs protein fractionation by SDS-PAGE be obtained 13 proteins with a molecular weight of 125.5; 100.9; 70.2; 56.7; 45.6; 40.4; 35.3; 34.1; 29.5; 27.4; 24.9; 20.7 and 19.7 kDa and can be seen in Figure 1.

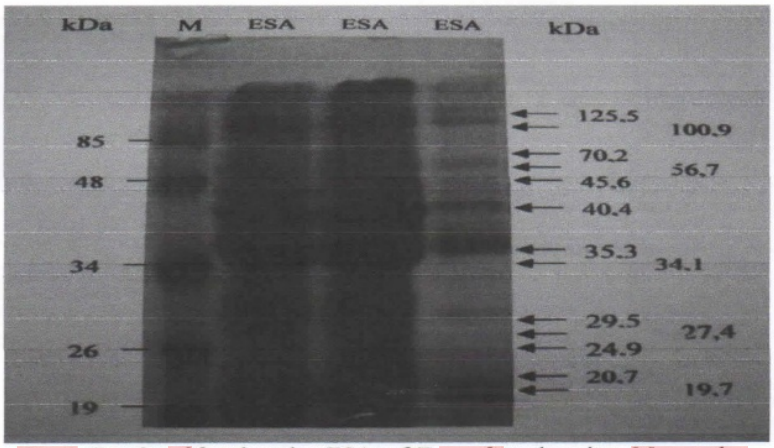

Fig. 1: Results of fractionation ESAs of *T. gondii* tachyzoites. M = marker.

Results of the determination of the antigenic proteins ESA using western blot is shown in Figure 2.

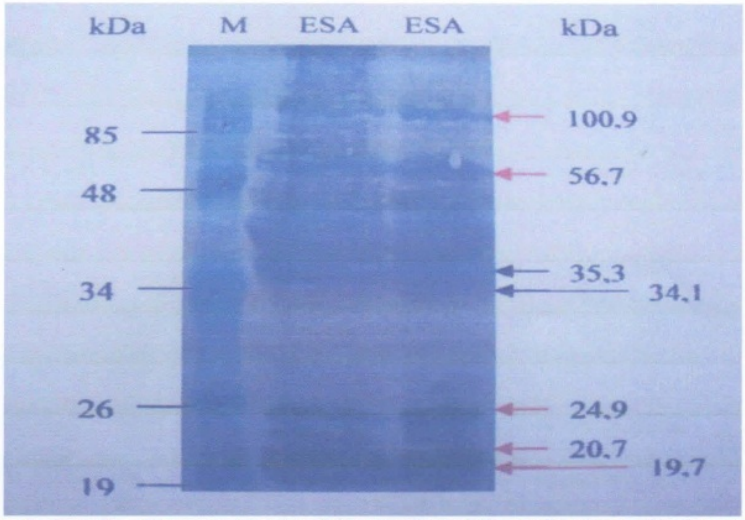

Fig. 2: Results of immunoblotting ESAs roteins of *T. gondii* with antibody anti ESAs. M = Marker ← = minor antigenic proteins, ← = major antigenic proteins.

Figure 2 showed 7 of the antigenic proteins were 100.9; 56.7; 35.3; 34.1; 24.9; 20.7 and 19.7 kDa, and 100.9; 56.7; 24, 9; 20.7 and 19.7 kDa were major antigenic proteins with a thicker ribbon results compared to the others.

Discussion

Excretory-secretory antigens of *T. gondii* is a protein that is released during the development of *T. gondii* both secreted (used for development) and excreted out of the cell. Protein is used for the process of attachment, penetration and multiplication. Joiner and Roos (2002) said that the tachyzoite of *T. gondii* have at least 3 organelles important as secretory organelles that micronema, rhoptry and dense granules. Micronema and rhoptry found on the anterior part of the cell being spread evenly dense granules in the cytoplasm. Micronema as exocytosis through attachment to host cells, secretion of rhoptry for invasion, and more dense granule secretion plays a role after parasite entry into host cells. Rhoptry and micronema protein are an important protein in the invasion of host cells, and dense granule protein required for intracellular replication including maintaining parasitophorous vacuole where the parasite is to lyse the host cells. Donahue *et al*., (2000) and Miller *et al*., (2001) reported that 15 proteins were expressed micronema TgMIC1 until TgMIC12, TgAMA1, TgM2AP and TgSUB1. Harper *et al*., (2004) and Barragan *et al*., (2005) said that the majority of these proteins function as adhesive proteins (protein for attachment), which was an essential protein for cell lysis invasion process cycle landlady. Strong attachment was needed so that the parasite gets into the cell, occurs invagination, and parasitophorous vacuoles form. ESAs proteins besides micronema protein is a protein that was expressed rhoptry. Ajioka and Soldati (2007) reported 24 proteins that were expressed rhoptry. Rhoptry protein is protein function penetration into the cell when the landlady like protein with a molecular weight of 43 kDa (ROP1), in addition there was some as yet unknown function. Granule proteins (GRA) is an important protein in the vacuole parasitophorous modification and function. Approximately 11 proteins that were expressed GRA of *T. gondii* tachyzoites and were mostly associated with the vacuole membrane of parasitophorous.

Performed repeated washing with a solution of the same composition with reduced ethanol and acetic acid addition of half of the previous 30 minutes. Further washing with a solution of 10% glutaraldehyde for 30 minutes and distilled. After washing the gel stained with AgNO3 for 15 minutes, then do the washing with distilled water twice each for 2 minutes. Given color development solution consisting of 3.7% formaldehyde, 5% zitronsauce and distilled water. After the protein bands visible then the reaction was stopped by adding 10% acetic acid. Results of gel that has seemed protein bands stored in 10% glycerol solution and ready to be documented. Calculation is done by comparing the molecular weight standard (mufasirin, 1999).

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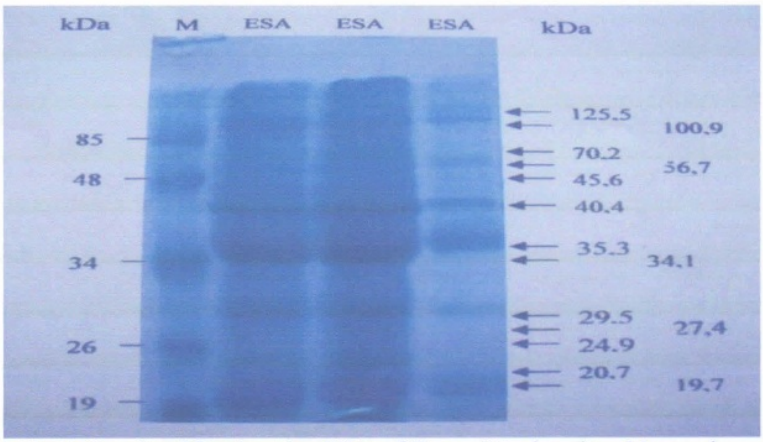

Fig. 1: Results of fractionation ESAs of *T. gondii* tachyzoites. M = marker.

The detection of protein by immunoblotting technique using immunological reaction using specific antibodies against the protein. Results of major proteins that were antigenic expected to be used for immunization process that were expected to protect the infection of *T. gondii* from the environment. Golkar *et al.,* (2005) reported the use GRA2 protein in mice and these proteins are immunogenic. Bivas-Benita GRA1 reported that protein was able to induce an immune response that was able to fight infection of *T. gondii* in animals because it could generate cellular and humoral immune. Vercammen *et al.,* (2000) reported that secreted GRA1 proteins from tachyzoite and bradyzoite able to evoke humoral immune response in mice and humans. According to Scorza *et al.,* (2003), gra-1 DNA vaccine can generate CD8 + T cells played a role which is very controlling acute infection of the *T. gondii*. Dalvani *et al.,* 2006 reported the use of ESAs was able to protect mice against challenge infection of *T. gondii*. Vercammen *et al.,* (2000) reported that the protein GRA1, GRA7 and ROP2 able to generate cellular and humoral immune response in mice. Vaccination with the protein able to reduce mortality in the acute phase but also able to inhibit parasite growth in chronic phase. Some researchers have tried to look at the potential of excretory-secretory proteins of dense-granule (GRA1) (Scorza *et al.,* 2003), GRA4 (Desolme, *et al.,* 2000), protein ROP2 (Leyva and Saavedra, 2001) and ROP1 (Chen *et al.,* 2001). The results showed that different protection against toxoplasmosis in mice. Among the existing vaccine candidates, MIC3 micronema protein (90 kDa) protein that was part of ESAs indicated a high potential due to it was inherent nature strongly on host cells either on tachyzoite, bradyzoite and sporozoite of *T. gondii* (Garcia-Reguet *et al.,* 2000; Cerede *et al.,* 2002). To know the ESAs were protective against infection *T. gondii* is necessary vaccination with the protein and performed to obtain ESA challenge test that can be used for the development of subunit vaccines toxoplasmosis.

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