

Cloning Gene Encoding Micronema 3 (Mic3) Protein of Tachyzoite *Toxoplasma Gondii* Local Isolate

Wayan T. Artama^{1,4*} Ni Nyoman Ayu Dewi^{1,2}, and Didik Tulus Subekti³

1. Research Center for Biotechnology, Gadjah Mada University, Yogyakarta 55281, Indonesia.
2. Faculty of Medicine Udayana University, Denpasar 80114, Indonesia.
3. BALITVET, Bogor, Indonesia.
4. Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia.

Abstract

Microneme 3 (MIC3) protein tachyzoites *Toxoplasma gondii* is one of protein which plays an important role during cell host invasion. Gene encoding MIC3 protein has been studied and it was suggested a potent vaccine candidate against *Toxoplasma gondii* infection. The aim of this research is to clone and sequence the gene encoding MIC3 protein of tachyzoites *Toxoplasma gondii* local isolate by amplification using polymerase chain reaction with specific primers. The amplified DNA fragment was cloned into pGEM-T and transformed into *E. coli* XL-1 Blue by heat shock method. Recombinant plasmids were isolated using alkali lysis method and analyzed by digestion using restriction endonuclease enzymes *Pst*I, *Hind*III, *Nco*I and *Eco*RV. The recombinant plasmids then sequenced to find out the nucleotide sequence of insert gene by ABIPRISM 377 DNA Sequencer. The DNA sequence then were analyzed by computer software for alignment. The result showed that transformation in *E. coli* XL-1 Blue by pGEM-T produced one clone that was encoding MIC3 protein. Analysis of 489 bp from 5' and 447 from 3' of gene sequence showed 97-98% homology with gene encoding for MIC3 protein of RH isolate.

Keywords: MIC3 protein, *Toxoplasma gondii*, tachyzoite, recombinant DNA

Introduction

Infection by *Toxoplasma gondii* are widely prevalent in animals and human all over the world. *Toxoplasma gondii* is an obligate intracellular protozoan parasite that infects all warm-blooded animals, including human, belonging to the phylum Apicomplexa. Other members of this phylum include the human pathogens *Plasmodium* and *Cryptosporidium* as well as the animals pathogens *Eimeria* and *Sarcocystis*. Cat and members of the cat family (*Felidae*) are the definitive hosts of *Toxoplasma gondii*, with many mammals and birds serving as intermediate hosts. Animals

and human acquire *Toxoplasma* by ingesting any of three infectious stages of the parasite: The rapid multiplying forms called tachyzoites, silent forms that occupy cysts in infected tissue called bradyzoites and oocysts shed in feces of infected cats. For human, infection could be ingestion of tissue cysts in under cooked meat and ingestion of water or food contaminated with oocysts. Widespread natural infection is possible because cats may excrete millions of oocysts after ingesting only a few tissue cysts from the infected animals. *Toxoplasma gondii* infects about 10%- 25% of world's population, more than 500 million people infected by *Toxoplasma gondii*. *Toxoplasma gondii* often cause subclinical infection or asymptomatic, but primary infection during pregnancy can lead to miscarriage and congenital defects (Gandahusada, 1998;

*Corresponding author: Wayan T. Artama, Research Center for Biotechnology, Gadjah Mada University, Jl. Teknik Utara, Berek, Sleman, Yogyakarta 55281, Indonesia. Tel: 62-274-564305; Fax: 62-274-520842; E-mail: artama@ugm.ac.id

Vercammen *et al.*, 2000). In immunosuppressed patients, this relative benign infection may reactivate and exhibit severe symptom including encephalitis, myocarditis, hepatitis and multisystem organ failure (Scorza *et al.*, 2003). Serological tests can be used to diagnose during acute toxoplasmosis but not always give accurate results (Gandahasada, 1998). Pyrimetamine and sulfonamide combination so far can use to treat toxoplasmosis case. These medications only affect to the tachyzoite but not the cyst form, so it can overcome acute infection but not chronic infection which can reactivate again (Gandahasada, 1998). Vaccine for toxoplasmosis is important to prevent fetal infections, reactivation in immunocompromised patients, and control tachyzoite multiplication which associated with the acute primary infection (Denkers *et al.*, 1998; Sibley *et al.*, 1996). Thus, biological molecular development diagnostic kit and vaccine is an important thing to do in an attempt to overcome these problems.

Attenuated *Toxoplasma gondii* tachyzoite vaccines have been successfully employed for animal use, but vaccination with live organisms cannot be safely performed in humans. The approach to a human toxoplasmosis vaccine must be based on the use of recombinant antigens or synthetic peptides that, ideally should protect the host from the all life cycle stages of the parasite *Toxoplasma gondii* (Prigione *et al.*, 2000). In the investigation of parasite antigens involved in protective immunity, most of the work had been focused on surface antigen (SAGs), specifically which expressed at the tachyzoite stage with a particular interest in SAG1 (Prigione *et al.*, 2000). Reports on experimental DNA vaccination in mice have been accumulating, and the antigens that have been tested now include membrane-associated surface antigen (SAG1), excreted-secreted dense granule proteins; GRA1, GRA4, GRA7 which expressed in both

tachyzoites and bradyzoites stages, as well as *rhoptry proteins*; ROP1 and ROP2 (Scorza *et al.*, 2003; Prigione *et al.*, 2000). The micronemal protein MIC3 looks particularly promising because it is a potent adhesion of *Toxoplasma gondii*, that is expressed in all three infectious stages of *Toxoplasma gondii* (tachyzoites, bradyzoites, and sporozoites) and that elicits early and powerful immune responses in mice (Ismael *et al.*, 2003).

A key step in *Toxoplasma gondii* infection is host cell invasion. Invasion is concomitant with the sequential discharge of proteins from the two secretory organelles of the parasite: first micronemes and then rhoptries (Coppens and Joiner, 2001). MIC3 protein is one of protein which is secreted by microneme organel during invasion by exocytosis. MIC3 is a soluble dimeric 90 kDa protein, synthesized as 40 kDa precursors that are proteolytically processed to 38 kDa final product. It has a strong affinity to host cell surface and the ability to bind cell surface is played out by *chitin binding-like* (CBL) domain. This domain is responsible for the adhesion and that N-terminal pro-peptide cleavage and C-terminal dimer formation are needed to allow the expression of this binding property (Cerede *et al.*, 2002; 2005; Black *et al.*, 2000). Gene encoding MIC3 protein has 2247 bp in length and contain no intron (Garcia-Reguet *et al.*, 2000).

In this study, we tried to clone gene encoding MIC3 protein tachyzoite *Toxoplasma gondii* from local isolate. Isolation of tachyzoite DNA was done after *in vivo* cultivation in Balb/C mice and then amplified using specific primers. The amplified DNA fragment then was ligated into pGEM-T vector and transformed into *Escherichia coli*, so we had only clones that carried out gene encoding MIC3 protein of local isolate, hopefully. Recombinant plasmids then were sequenced and identified its homolog with gene encoding MIC3 of RH isolate.

Materials and Methods

Isolation of tachyzoite DNA

After parasite *in vivo* cultivation in Balb/C mice and obtained 6×10^8 tachyzoite/ml, intraperitoneal fluid was centrifuged and the pellet was washed three times using PBS then resuspended with NTE solution into the resuspended pellet. Added with 100 µg/ml proteinase-K and 0.5% SDS, afterward overnight incubated in waterbath 37°C. This reaction then was added with an equal volume of phenol, mixed by shaking 60 rpm for 20 min, centrifuged at 3,000 rpm for 15 min. The aqueous phase was added with an equal volume of CIAA and centrifuged at 3,000 rpm for 10 min. This step was repeated until there was no interphase. Finally, DNA was precipitated with 0.1 volume 3 M Na-acetate and 2x volume ice-cold ethanol absolute. Incubated for 15 min in -20°C, centrifuged at maximum speed for 5 min. Rinsed the pellet with 70% ethanol and dissolved in TE buffer.

Amplification of target DNA

Specific primers for gene encoding MIC3 protein, M3F1 and M3R1 were diluted into 10 pmol/µl (M3F1: 5' GTGTAAGCTTC TTGTTCAACTGGGTA 3' and M3R1: 5' CACGGATATCTGCGAATGGGCG 3'). Amplification was done using *puRe Taq RTG-PCR*. The amplification mixture then amplified using *thermocycler (Gene Cycler, Biorad)* with program: (1) initial denaturation at 94°C for 5 min, (2) denaturation at 94°C for 1 min, (3) annealing at 60°C for 1 min, (4) extension at 72°C for 1 min, (5) repeat the cycle for 35 times, and (7) final extension at 72°C for 5 min. Amplification was done with positive control (RH isolate) and negative control.

Cloning and transformation

The amplified of DNA fragment was ligated into pGEM-T vector (Promega) according to recommended procedure.

Transformation into *E. coli* XL-1 Blue was done using heat shock method at 42°C for 90 s. Transformed *E. coli* then was plated on LB agar containing ampicillin by adding, IPTG, and X-gal. Plasmid from transformed *E. coli* then was isolated by alkali method and analyzed by digestion using endonuclease restriction enzymes (*Pst*I for plasmid from blue colonies, *Hind*III for plasmid from white colonies and double digest using *Eco*RV and *Nco*I to show the existence of the insert).

Sequencing of recombinant plasmids

The recombinant plasmid was sequenced using Big Dye Terminator ABIPRISM 377 DNA Sequencer (Eijkman, Jakarta). Sequence results were aligned using BLAST software program which was accessed through NCBI at <http://www.ncbi.nlm.nih.gov>.

Results and Discussion

Amplification of target DNA

The entire gene encoding MIC3 is 2247 bp in length, according to gene bank (NCBI) accession no. AJ132530. M3F1 primer annealed from position 666 to 692 and M3R1 from 1812 to 1833; both of them were designed to amplify gene target for 1170 bp (position 666 to 1833), including signal sequence (position 696 to 773) and mature peptide (position 894 to 1772). The sequence of M3F1 was modified to generate *Hind*III restriction site and M3R1 for *Eco*RV restriction site (Figure 1).

These restriction sites later are used to digest recombinant plasmids. The result of DNA amplification using specific primers can be seen at Figure 2, showed that was one dominant band at approximately 1.2 kb at positive control (RH isolate) and sample (local isolate), either and there was no bands at negative control. But there was one thin band at 300 bp and seemed to primer dimer.



Figure 1. Construct of M3F1 and M3R1 primers.

Ismael *et al.* (2003) amplified gene encoding MIC3 from pBluescript II SK-MIC3 using same primers and obtained amplicon at 1155 bp.

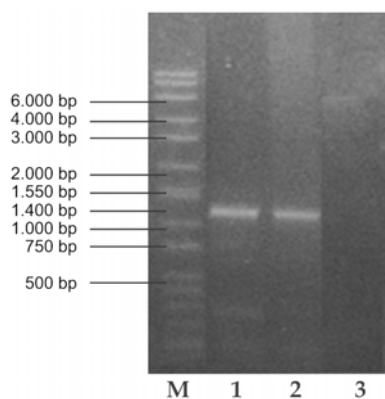


Figure 2. Amplification of DNA using M3F1 and M3R1 primers on 1% agarose gel. (M) marker, (1) positive control (RH isolate), (2) sample (local isolate), (3) negative control.

Cloning and transformation

We obtained 8 colonies on 1 plate LB agar. They consist of 2 blue colonies and 6 white colonies (data not shown). The plasmids from transformed *E. coli* then were isolated and digested with *Pst*I (for plasmids from blue colonies) and *Hind*III (for plasmids from white colonies). Restriction site for *Pst*I was available at polycloning sites of pGEM-T

vector and site for *Hind*III was appropriated from construct of M3F1 primer; pGEM-T did not have this site (Circular map of pGEM®-T vector (Technical Manual No. 042, Promega). Digested plasmids from blue colonies were 3 kb in length (suitable to pGEM-T) and only 1 plasmid from white colonies had 4.2 kb size as we desire (data not shown). This 4.2 kb plasmid then named with pWTA-M3.

Digestion using *Hind*III and *Pst*I was performed to show the existence of 1.2 kb insert but we could not get the result. pWTA-M3 was linearized with *Hind*III. It was 4.2 kb but after digested with *Pst*I became 3 kb and there were bands at approximately 300-400 bp. Perhaps, it caused by too many restriction sites for *Pst*I in insert gene, thus the insert was digested into many smaller fragments. In an attempt to overcome this problem, we did *double digest* using *Nco*I and *Eco*RV. We had 4.2 kb plasmid after linearized with *Hind*III and insert at 1 kb after double digested (Figure 4). This smaller insert (must be 1.2 kb) may due to there was another site for *Nco*I in insert gene.

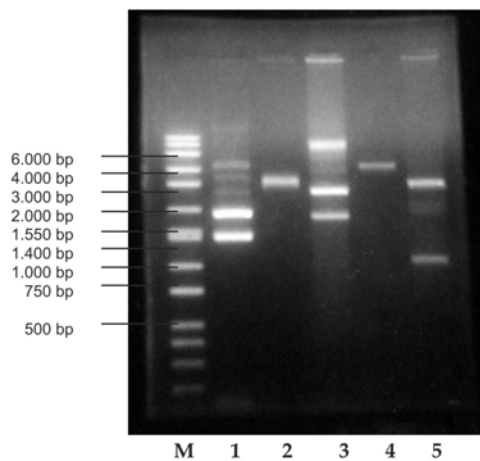


Figure 4. Plasmid digestion using endonuclease restriction enzymes on 1% agarose gel. (M) marker; Plasmid from blue colony (1) whole plasmid, (2) digested with *Pst*I; pWTA-M3 (3) whole plasmid, (4) digested with *Hind*III, (5) digested with *Nco*I and *Eco*RV.

Sequencing of recombinant plasmids

Sequencing of pWTA-M3 was done with *pUC/M13 forward sequencing primer* and *pUC/M13 reverse sequencing primer* (available at pGEM-T vector construct). We had 489 bp from 5' and 447 bp from 3'; so there were 234 bp from 1170 of insert gene that could not know its nucleotide sequence.

The analysis of the DNA sequence obtained from the BLAST program revealed a significant degree with gene encoding MIC3 *Toxoplasma gondii* RH isolate. Sequence alignment of pWTA-M3 which sequenced using *pUC/M13 forward* showed 97% homolog from base 676 until 1164, the differences were lied on position 1074, 1075, 1129, and 1160. Alignment of pWTA-M3 sequence which sequenced using *pUC/M13 reverse* showed 98% homolog from base 1387-1833, the differences were lied on base 1827 and 1825. The difference bases at 1074 and 1075 lead to alter amino acid; aspartic acid (GAT) to glycine (GGA), difference at 1129 did not change the amino acid (CTG to CTT:

leusin), and difference at 1160 changed aspartic acid (GAC) to glutamic acid (GAA). Un homolog sequence at base 1825 and 1827 were not in *coding sequence* of gene encoding MIC3 so did not affect the amino acid synthesis. These changes may have no effect to the general structure and functional of the protein because aspartic acid and glutamic acid have similar property (polar/hydrophilic) and similar size, and so do glycine, have polar/hydrophilic property; it just has a smaller size cause its side chain only hydrogen (Berg *et al.*, 2002).

Analyze of insert gene in pWTA-M3 using ChromasPro showed that it had many restriction sites for endonuclease restriction enzymes include *Pst*I dan *Nco*I; it could explain the problems when recombinant plasmids analysis. The insert gene had 2 sites for *Pst*I (CTGCAG) at position 1016-1021 and 1424-1429 thus recombinant plasmids were digested into 4 fragments; 1 fragment with 3 kb and 3 fragments with size at approximately 400 bp. Double digested using *Nco*I and *Eco*RV showed a smaller insert because that was 1 site for *Nco*I (CCATGG) at position 1646-1651 so the plasmids were showed into 3 fragments with 3 kb and 1 kb fragment at approximately 200 kb.

Acknowledgments

This research was financially supported by Riset Unggulan Terpadu (RUT) XI and the authors were appreciated the helpful suggestion of Prof. Dr. Sudjadi and Dr. Widya Asmara.

References

- Berg, J. M., Tymoczko, J. L. and Stryer, L., 2002. *Biochemistry*. 5th ed. New York: W. H. Freeman and Company, 41-73, 134-135.
- Black, M.W. and Boothroyd, J.C., 2000. Lytic cycle of *Toxoplasma gondii*. *Microbiol. Mol. Biol. Rev.*, **64** (3), 607-623.

- Cerede, O., Dubremetz, J. F., Bout, D. and Lebrun, M., 2002. The *Toxoplasma gondii* protein MIC3 requires pro-peptide cleavage and dimerization to function as adhesins. *EMBO J.*, **21** (11), 2526-2536.
- Cerede, O., Dubremetz, J.F., Soete, M., Deslee, D., Vial, H., Bout, D. and Lebrun, M., 2005. Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence. *J. Exp. Med.*, **201** (3), 453-463.
- Coppens, I. and Joiner, K.A., 2001. Parasite-host cell interactions in toxoplasmosis: new avenues for intervention? *Expert review in molecular medicine*. [e-book]. Cambridge University Press. Available from: <http://www-ermm.cbcu.cam.ac.uk>
- Denkers, E.Y. and Gazzinelli, R.T., 1998. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin. Microbiol. Rev.*, **11** (4), 569-588.
- Gandahusada, S., 1998. *Toxoplasma gondii*. In Gandahusada, S., Ilahude, H.H.D., and Pribadi, W., eds. *Parasitologi Kedokteran*. Edisi ketiga. Jakarta: Balai Penerbit FK UI, 153-161.
- Garcia-Reguet, N., Lebrun, M., Fourmaux, M.N., Mercereau-Puijalon, O., Mann, T., Beckers, C.J.M., Samyn, B., Beeumen, J.V., Bout, D. and Dubremetz, J.F., 2000. The microneme protein MIC3 of *Toxoplasma gondii* is a secretory adhesin that binds to both the surface of the host cells and the surface of the parasite. *Cell. Microbiol.*, **2** (4), 353-364.
- Ismael, A.B., Sekkai, D., Colli, C., Bout, D. and Mevelec, M.N., 2003. The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. *Infect. Immun.*, **71** (11), 6222-6228.
- Prigione, I., Facchetti, P., Lecordier, L., Deslee, D., Chiesa, S., Cesbron-Delauw, M.F. and Pistoia, V., 2000. T-cell clones raised from chronically infected healthy humans by stimulation with *Toxoplasma gondii* excretory-secretory antigens cross-react with live tachyzoites: characterization of the fine antigenic specificity of the clones and implications for vaccine development. *J. Immunol.*, **164**, 3741-3748.
- Scorza, T., Souza, D., Laloup, M., Dewit, J., Braekeleer, J.D., Verschueren, H., Vercammen, M., Huygen, K. and Jongert, E., 2003. A GRA1 DNA vaccine primes cytolytic CD8+ T cells to control acute *Toxoplasma gondii* infection. *Infect. Immun.*, **71** (1), 309-316.
- Sibley, L.D. and Howe, D.K., 1996. Genetic basis of pathogenicity in toxoplasmosis. In Gross, U., ed. *Current Topics in Microbiology and Immunology*. Berlin, Heidelberg, Springer-Verlag. 3-13.
- Vercammen, M., Scorza, T., Huygen, K., Braekeleer, J.D., Diet, R., Jacobs, D., Saman, E. and Verschueren, H., 2000. DNA vaccination with gene encoding *Toxoplasma gondii* antigens GRA1, GRA7, and ROP2 induces partially protective immunity against lethal challenge in mice. *Infect. Immun.*, **68** (1), 38-45.