Journal of Animal Research: v.6 n.6, p. 957-960. December 2016

DOI: 10.5958/2277-940X.2016.00137.6

Molecular Cloning and Characterization of Microneme Gene 10 (MIC10) of Kolkata Isolate of *Toxoplama gondii*

Raghubir Singh[1]*, Muthu Sankar[2], Navjot Singh[3], Utpal Kumar Chattopadhyay[1], Mohd. Ashraf Malik[3] and Chanchal Singh[4]

[1]All India Institute of Hygiene and Public Health, Kolkata, INDIA
[2]Temperate Animal Husbandry, Indian Veterinary Research Institute, Mukteshwar, INDIA
[3]Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, INDIA
[4]Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, INDIA

**Corresponding author: R Singh; Email: drraghubir166@gmail.com*

Received: 03 June, 2016 **Accepted:** 15 September, 2016

ABSTRACT

The present study dealt with cloning and molecular characterization of MIC10 gene of *T. gondii* Kolkata isolate. TgMIC10 is a small microneme protein that lacks a putative transmembrane domain and appears not to remain associated with the parasite membrane after micronemal discharge. MIC10 gene of *T. gondii* "Kolkata isolate was PCR amplified and cloned into a TA cloning vector (Promega) to facilitate sequencing and subsequent characterization. The product revealed 597bp in size and has 99.2% homology with the published sequence. The difference was observed at 22, 210, 235, 378 and 513 positions. When the gene was deduced for amino acids, the homology was 98% with published sequence. The total amino acids number was 198 with predicted mass of 23 kDa molecular weight. The changes were observed at 8, 115 and 171th amino acid positions.

Keywords: *Toxoplasma gondii*, MIC10, TA cloning vector

Toxoplasma gondii is an obligate intracellular protozoan parasite with a broad host range involving both mammals and birds. Amongst the apicomplexans, *T. gondii* is widely prevalent and has been reported from all the continents. Felines serve as definitive hosts, while all warm blooded animals act as intermediate hosts of the parasite with disseminated tissue infections (Tenter *et al*., 2000). Micronemes are specialized apical secretory organelles, located immediately beneath the cell membrane near the anterior end of the apical and presumed to play a predominant role in the early phase of the invasion process by discharging adhesins capable of interacting with host cell receptors (Hoff *et al*., 2001). TgMIC10 is a small microneme protein lacking a putative trans-membrane domain. Indirect immune-fluorescence microscopy revealed that it fails to remain associated with the parasite membrane after microneme discharge (Hoff *et al*., 2001). This raises the possibility that it could diffuse from the point of parasite invasion in the tissues and become accessible as a circulating antigen. In light of the relatively high level of TgMIC10 expression in tachyzoites, the stage responsible for active infection, direct detection of TgMIC10 present in bradyzoites and tachyzoites of *T. gondii* may be useful to differentiate between active and latent infection (Hoff *et al*., 2001). The present study encoded the DNA sequence fragment of *T. gondii*, Kolkata isolate MIC10 proteins using the recombinant technique. The DNA of Kolkata isolated was being used for PCR amplification for MIC10 gene and cloned into a TA cloning vector (Promega) to facilitate sequencing and subsequent characterization for a product of 597bp size.

MATERIALS AND METHODS

Cloning

The extraction of genomic DNA was carried out from the oocysts of the Kolkata isolate of *T. gondii* following

the technique outlined by Dubey *et al*., 2004. The purity of DNA was checked by ethidium bromide stained agarose gel electrophoresis using a submarine horizontal electrophoresis apparatus (Bangalore Genei). The concentration of double stranded DNA was calculated from the A260 value using the formula: concentration of DNA (µg/µl) = A260 × 50. The working concentration of 10ng/µl was prepared by diluting the stock solution in water for application in PCR.

PCR based amplification of the entire ORF of MIC-10 gene of *T. gondii* (Kolkata) was carried out by using the self designed specific pair of forward and reverse primers. MIC10-F-5CCG GTC GAC ATG GCG CTT TCT TCT TTG AAC3 and MIC10-R-5CCG AAG CTT CTA CAT TGA TTT CCT GCG TC3 were laboratory designed and custom synthesized by using the published complete sequence for TgMIC10 (GenBank Accession No. AF293654). The PCR reaction was performed in a total volume of 25 µl containing 0.5 µl of genomic DNA, 1.5 µl of each primers (F and R), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl dNTP, 2.5 µl of 10x dream *Taq* buffer, 16 µl NFW and 1 unit of *Taq* DNA polymerase. This solution was initially denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 40 s, 59°C for 40 s, 72°C for 1 min, with a final extension step at 72°C for 10 min. The positive amplification of the target sequence was visualized by gel-electrophoresis of the PCR product at 1.5% agarose gel stained with ethidium bromide in a horizontal electrophoresis apparatus.

The PCR product (obtained from Kolkata isolate) was purified using QIAGEN Mini elute gel extraction kit following manufacturer's protocol. The quantification of the purified PCR product was done spectrophoto metrically (Nanodrop®, USA). The PCR amplified DNA sequence coding for MIC10, thus purified, and was used for cloning into TA clone PCR cloning vector (Promega). Ligation reaction for cloning of MIC10 into TA clone PCR cloning vector was carried out as follows:

Cloning vector	1.0 µl (50 ng)
Purified PCR product (MIC10)	3.0 µl (115 ng)
2X ligation buffer	5.0 µl
T4 DNA ligase	1.0 µl
Total	10.0 µl

The ligation reaction was allowed to continue at 4°C for overnight.

Transformation of DH5-α cells

An aliquot (250 µl) of the frozen competent cells was thawed on ice for 30 min. To this 5 µl of the ligation mixture was added and mixed gently and then further incubated on ice for 15-20 min. The cells were then subjected to heat shock at 42°C for 90 sec, and immediately chilled on ice for 10 min. The cells were supplemented with 800 ml of fresh autoclaved LB broth and incubated for 1 h at 37°C. Two hundred microlitres of the transformed cells were then plated on LB agar plates supplemented with ampicillin (100 µg/ml), X-gal (30 µg/ml) and IPTG (0.5 mM/ml). The plates were then allowed to dry under laminar flow and subsequently incubated at 37°C for 12 -14 h for the development of blue and white colonies.

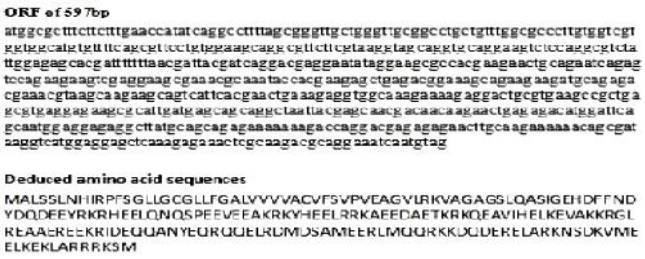

Fig. 1: Nucleotide sequence and amino acid sequence of MIC10 gene of *T. gondii* (Kolkata strain)

Colony screening and Characterization

In order to identify the positive clones (i.e. containing the inserts), plasmids were isolated from the white colonies and digested with restriction enzyme *Eco*RI. Plasmid DNA from the transformed DH5α cells was isolated following standard protocol (Sambrook *et al*., 1989). A subculture of positive clone harbouring the desired MIC10 gene was custom DNA sequenced for nucleotides from Department of Biochemistry, Delhi University. The sequence information received was analyzed using DNASTAR and GeneTool software.

RESULTS AND DISCUSSION

The isolated DNA from the *T. gondii* oocysts was found intact and without smearing in gel electrophoresis. The purity of DNA was checked by measuring $OD_{260/280}$ in a spectrophotometer. At 1.86 the concentration of DNA was 114ng/µl. PCR amplification of MIC-10 gene resulted

in 597bp amplified product (Fig. 2A). The previous study used the tachyzoites cDNA of *T. gondii* RH strain to characterize the TgMIC10 gene revealed it being 597bp and lacks homology to any previously characterized proteins and has an unusually long secretory leader sequence of 58 amino acids with molecular weight of 18 kDa, possesses nine diglutamic acid repeats and an imperfect repeat sequence (RK(R/Y) HEEL and is entirely devoid of cysteines by Hoff *et al.* (2001).

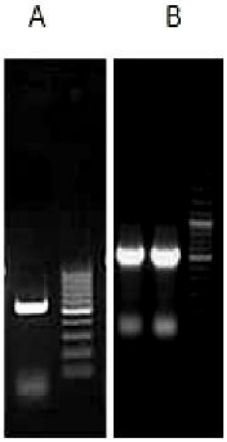

Fig. 2: Amplified PCR product and plasmid of MIC10 gene of *T. gondii* (Kolkata isolate)

(A) PCR amplification, Lane 1: 100 bp marker (Fermentas), Lane 2: MIC-10 gene (597 bp).
(B) Plasmid PCR, Lane 1: 100 bp marker (Fermentas), Lane 2, 3: MIC-10 gene (597 bp)

The present study cloned MIC-10 gene in a TA cloning vector to facilitate the sequencing and characterization. The white colonies as well as a few blue colonies were observed after incubation of the plate at 37 ºC for 12–16 hrs following ligation and transformation. Randomly, five white colonies were selected for checking and confirmation of the presence of the gene. The product of size 597 bp was amplified in culture PCR with the clones (Fig. 2B). The cloned ORF of the full length MIC-10 gene of *T. gondii* Kolkata isolate was sequenced using an automated DNA sequencer. Analysis of the sequence using the software DNA STAR, Laser gene, revealed an ORF of 597bp (Fig.1). The Kolkata isolate showed 99.2% homology with published sequence of *T. gondii* RH strain (GenBank Accession No. AF293654). The differences were observed at 22, 210, 235, 378 and 513 positions. When the gene was deduced for amino acids, the homology was 98%. The total amino acids number was 198 with 23 kDa molecular weight. The changes were observed 8, 115 and 171.

The three published microneme proteins of *Toxoplasma gondii* RH strain *viz.*, TgMIC1 (Achbarou *et al.*, 1991; Fourmaux *et al.*, 1996), TgMIC2 (Wan *et al.*, 1997), and TgMIC3 (Achbarou *et al.*, 1991) and other putative microneme proteins (TgMIC's 4 and 6–9) contain one or more adhesive motiffs. However, recently, two more microneme proteins of *T. gondii* were discovered which did not possess recognizable adhesive motifs. The first member of this class is TgMIC5, it lacks adhesive domains and has homology to the parvulin family of peptidyl prolyl cis-trans isomerases (PPIases) (Brydges *et al.*, 2000). The second member TgMIC10 is a non-adhesive microneme proteins. Southern blot analysis of TgMIC10 of *T. gondii* RH strain has shown, being present as single copy in the parasite genome and an amenable target for gene replacement and assessment of mutant phenotype (Hoff *et al.*, 2001). MIC10 Kolkata isolate showing the homology with the published strain, thus, could act as equally potent strain for further study on this gene. Expression of microneme genes including MIC10 by recombinant DNA technology may prove to be helpful for a safe, cheap and potent local antigens for diagnostic and prophylactic point of view to protect our native population for both humans as well as animals.

Protein details

% A = 33.33 [199]	Molecular Weight 23.12 kDa
% G = 30.65 [183]	198 Amino Acids
% T = 17.92 [107]	39 Strongly Basic(+) Amino Acids (K,R)
% C = 18.09 [108]	43 Strongly Acidic(-) Amino Acids (D,E)
% A+T = 51.26	58 Hydrophobic Amino Acids (A,I,L,F,W,V)
% C+G = 48.74	37 Polar Amino Acids (N,C,Q,S,T,Y)
	5.719 Isolectric Point

SUMMARY

Microneme are discharged at the time of host cell contact and is speculated that these proteins are involved in recognition of host cell receptors and active entry into the

target cell. Generation of a TgMIC10 mutant parasite line enable us to determine its role in the parasite life cycle in both in vivo and in vitro infections. The TgMIC10 regardless having a role in parasite invasion may be useful as a diagnostic tool and prophylactic purpose. In this study, DNA sequences encoding fragment of *T. gondii* MIC10 protein was cloned and characterized using Kolkata isolated of *T. gondii* revealed 597 bp in size and has 99.2% homology with published sequence and its expression in a prokaryotic vector could help in using it as an antigen for diagnostic and/or prophylactic use.

REFERENCES

Achbarou, A., Mercereau-Puijalon, O., Autherman, J.M., Fortier, B., Camus, D. and Dubremetz, J.F. 1991. Characterization of microneme proteins of *Toxoplasma gondii. Mol. Biochem. Parasit.*, **47**: 223–234.

Brydges, S.D., Sherman, G.D., Nockemann, S., Loyens, A., Daubener, W., Dubremetz, J. F. and Carruthers, V. B. 2000. Molecular characterization of TgMIC5, a proteolytically processed antigen secreted from the micronemes of *Toxoplasma gondii. Mol. Biochem. Parasit.*, **111**: 51–66.

Dubey, J. P. and Frenkel J. K. 1972. Cyst-induced toxoplasmosis in cats. *J. Protozool.*, **19**: 155–177.

Dubey, J.P., Navarro, I.T., Sreekumar, C., Dahl, E., Friere, R.L., Kawabata, H.H., Vianna, M.C., Kwok, O.C., Shen, S.K., Thulliez, P. and Lehman, T. 2004. *Toxoplasma gondii* infection in cats from Parana, Brazil: Seroprevalence, tissue distribution and biologic and genetic characterization of isolates. *J. Parasitol.*, **90**(4): 721-26.

Fourmaux, M.N., Achbarou, A., Mercereau-Puijalon, O., Biderre, C., Briche, I., Loyens, A., Odberg-Ferragut, C., Camus, D. and Dubremetz, J.-F. 1996. The MIC1 microneme protein of *Toxoplasma gondii* contains a duplicate receptor-like domain and binds to host cell surface. *Mol. Biochem. Parasit.*, **83**: 201–210.

Hoff, E.F., Cook, S.H., Sherman, G.D., Harper, J.M., Ferguson, D.J.P., Dubremetz, J.F. and Carruthers, V.B. 2001. *Toxoplasma gondii:* Molecular Cloning and Characterization of a Novel 18-kDa Secretory Antigen, TgMIC10. *Exp. Parasitol.*, **97**: 77-88.

Sambrook, J., Fritch, E.F. and Maniatis, T. 1989. *Molecular cloning: A laboratory manual, 2nd edition, Cold spring harbor Laboratory press.*

Tenter, A.M., Heckeroth, A.R. and Weiss, L.M. 2000. *Toxoplasma gondii*: from animals to human. *Int. J. Parasitol.*, **30**: 1217-1258.

Wan, K.L., Carruthers, V.B., Sibley, L.D. and Ajioka, J.W. 1997. Molecular characterization of an expressed sequence tag locus of *Toxoplasma gondii* encoding the micronemal protein MIC2. *Mol. Biochem. Parasit.*, **84**: 203–214.