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A *Toxoplasma gondii* 10 kDa *in vitro* excretory secretory antigen reactive with human IgM and IgA antibodies

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Abstract. Toxoplasmosis can cause serious disease in immunocompromised patients and to congenitally infected foetuses. Appropriate laboratory investigations in potential cases of acute *Toxoplasma* infection are important. Excretory secretory antigen (ESA) is immunogenic during both human and experimental infections, therefore is considered as a good candidate for investigation into new infection markers. In this study, ESA was prepared from *in vitro* cultures of *Toxoplasma gondii* to identify *T. gondii* ESA antigenic component(s) that is/are most reactive with serum samples from probable acute cases of toxoplasmosis. Serum samples were obtained from several categories of individuals with the following *Toxoplasma* serology: Group I: IgM+ IgG+ (low IgG avidity) or IgM+ IgG- from sera of patients who had clinical query of toxoplasmosis (n=35). Group II: IgM- IgG+ (high IgG avidity) from chronically infected individuals (n=30). Group III: normal/healthy individuals with anti-*Toxoplasma* IgM- IgG- (n=20). Group IV: individuals with other infections who had anti-*Toxoplasma* IgM- IgG- (n=10). The ESA was subjected to SDS-PAGE, followed by Western blot analysis using the above sera and probed with peroxidase conjugated anti-human IgM and IgA antibodies. The blots were then developed using chemiluminescence substrate. The selected antigenic band was excised from the gel after two dimensional electrophoresis and sent for mass spectrometry analysis using MALDI TOF-TOF. The most promising antigenic band was a 10 kDa protein which showed sensitivity of 80% in both IgM and IgA blots, and specificity of 96.7% with sera from other infections and healthy controls. The two best identifications for the 10 kDa band were ubiquitin (ribosomal protein CEP52 fusion protein) and polyubiquitin.

INTRODUCTION

Toxoplasmosis, caused by the intracellular protozoan parasite, *Toxoplasma gondii*, is ubiquitous throughout the world. In recent years, the significance of congenital toxoplasmosis has been increasingly recognized (Bhopale, 2003). The incidence of toxoplasmosis, especially toxoplasmic encephalitis, has risen with the increasing population of patients with AIDS (Luft & Remington, 1992), whereas patients with a variety of neoplastic diseases as well as patients receiving immunosuppressive therapy are at risk of reactivation of *T. gondii* infection (Israelski & Remington, 1993).

Laboratory diagnosis of *Toxoplasma* infection is usually based on the detection of specific antibodies. Many serological tests for the detection of *T. gondii* specific immunoglobulin are commercially available, however most of these commercial kits use native parasite antigen prepared from tachyzoites that might be contaminated by non-parasitic material (Pietkiewicz *et al.*, 2004). The replacement of recombinant antigens in place of tachyzoites might reduce the cost, improve reproducibility and reduce cross-reactions thus improving test specificity. Recently a number of recombinant *T. gondii* antigens have been prepared and applied in ELISA systems with

promising results. However it is still important to produce new recombinant antigens since a panel of tests is usually needed for diagnostic confirmation. Thus identification of new infection markers would be required before the corresponding recombinant antigens can be produced.

Secretion of compounds by the apical complex of *Toxoplasma* has been shown to be important for invasion, replication and survival of the parasite within host cells (Carruthers, 2002). There are three secretory organelles namely micronemes, rhoptries and dense granules. Micronemes are small rod-shaped structures, located in the most apical area of the parasite, and involved in recognition and adhesion to the host cell. Rhoptries, organized as a group of elongated, club-shaped organelles, are located at the anterior region that extend from within the conoid toward the nucleus. Rhoptry protein is secreted at the time of invasion and is associated with membrane of parasitophorous vacuoles and drive the invasion of the parasite into the host cell. Dense granules with spherical shape, are found throughout the cell but mostly in the posterior part of the parasite, and involved in the maturation of the parasitophorous vacuoles where the parasite multiplies (Morrissette & Sibley, 2002). Excretory secretory antigens constitute a major part of the circulating antigens in acutely infected animals or humans, thus is one of the first targets of the immune response. ESA of *T. gondii* has been reported to be useful in identifying potential diagnostic markers (Hafid *et al.*, 1992; Dedkova *et al.*, 2000; Ahn *et al.*, 2005; Rome *et al.*, 2008; Michelin *et al.*, 2009). When ESA was used as an antigen in enzyme immunoassay, it showed high sensitivity in the detection of IgM antigens in congenital toxoplasmosis (Dedkova *et al.*, 2000).

For ESA production, the RH strain of *T. gondii* can be easily grown *in vivo* and *in vitro*. However, antigen production via passage in mice is considered an expensive method. The inoculated mice survive infection for only 4-6 days and the tachyzoites are often contaminated with peritoneal cells. However propagation of tachyzoites in cell culture is more economical (Diab & El-Bahy, 2008), reliable and a consistent quality can be maintained. Thus the present study focused on investigation of the diagnostic potential of the excreted secreted antigen (ESA) of *in vitro* grown *T. gondii*.

MATERIALS AND METHODS

Preparation of excretory secretory antigens (ESA)

Previously, we have optimised the method for the cell culture of *T. gondii* tachyzoites that produced parasites with least contamination of host cells (Saadatnia *et al.*, 2010). VERO cells at 85% confluence in 75-cm^2 cell culture flasks (Nunclon, Roskilde, Denmark) were washed four times with 1x Phosphate buffered saline (PBS), followed by addition of Dulbecco's Modied Eagle's Medium (DMEM) (Gibco BRL, USA) containing 100 IU/ml penicillin and 100 µg/ml streptomycin) (Gibco BRL, USA) without fetal bovine serum (FBS), and then infected with $1x10^7$ tachyzoites. After 48 hours post-infection, the culture supernatant was harvested by centrifugation for 10 min at 1000g, filtered through a 0.22mm pore-size Millipore filter. Pooled *in vitro* ESA was concentrated using a stirred cell apparatus (Millipore, USA) at 4ºC. Gas pressure was applied directly to the ultrafiltration cell (<55 psi). Subsequently 40 µl/ml protease inhibitor cocktail (Roche, Germany) was added to the pooled ESA, and the protein concentration was determined by Bio-Rad protein assay (BioRad, USA). The pooled *in vitro* ESA was aliquoted into sterile microcentrifuge tubes and stored at -70ºC until use. Two control antigens were prepared, one in same manner as the culture flasks prepared for *T. gondii* ESA, but without infection with the parasite; and the second was the supernatant of sonicated host cell lysate.

Serum samples

Serum samples used in this study were part of our serum bank and collected according to the requirements of USM research ethics committee. Sera were classified based on clinical information and laboratory diagnosis

for toxoplasmosis. Toxoplasma IgM, IgG and IgG-avidity were determined using commercial kits (Platelia, Bio-Rad, France). The serum samples were grouped as follows: Group I: IgM+ IgG+ (low IgG avidity) or IgM+ IgG– from sera of patients who had clinical query of toxoplasmosis (n=30). Group II: IgM– IgG+ (high IgG avidity) from chronically infected individuals (n=30). Group III: normal/ healthy individuals with anti-*Toxoplasma* IgM- IgG- (n=20). Group IV: individuals with other infections who had anti-*Toxoplasma* IgM- IgG- (n=10). i.e. malaria, leptospirosis, dengue, filariasis and amoebic liver abscess.

SDS-PAGE and Western blotting

Pooled ESA at 20 µg per well was dissolved in sample buffer (1:1 ratio) containing 28.6% SDS and 4.76% 2- mercaptoethanol, boiled for 10 minutes and then spun for 3 minutes at 12000 *g*. It was then subjected to 12% SDS-PAGE using Mini- Protein 3 cell apparatus (Biorad, USA) in running buffer (pH 8.3) containing 0.3% tris, 1.44% glycine and 0.1% SDS. Electrophoresis was run at room temperature (RT) with a constant current of 100 V for 90 minutes. Transfer buffer was prepared from a mixture of 0.3% tris, 1.44% glycine and 20% methanol. The gel was then transferred to a nitrocellulose paper (NCP), 0.45µm by using a Trans-Blot Cell (Bio-Rad USA) and was run at a constant current of 12 A for 30 minutes. After the transfer, unbound sites on NCP were blocked with 1% blocking reagent (Roche Diagnostics, Germany) for one hour, RT. The NCP was subsequently washed 3 times at 15, 10 and 5 minutes interval in washing buffer (Tris- HCL buffered saline- 0.05% Tween 20, TBST); cut into strips, followed by incubation overnight at 4°C with diluted serum. Rheumatoid factors (RF) absorbent (SERION, Germany) was added to remove rheumatoid factors when performing IgM Western blots. After another washing step, the diluted secondary antibody conjugates were added i.e. monoclonal anti-human IgM and IgA conjugated to HRP (Zymed, USA). After 1 hour incubation, the strips were washed again and subsequently developed using BM chemiluminescence blotting substrate (Roche Diagnosrtic, Germany) and Kodak films (Kodak, USA). To establish the optimal working dilution for serum samples (primary antibody) three different dilutions (1:25, 1:50 and 1:100) were performed. For secondary antibody, dilutions of 1:1000, 1:2000, 1:4000 and 1:8000 were tested. The molecular weights of proteins specific to *T. gondii* were based on a Western blot standard marker (Precision Plus Protein standard, unstained marker, BioRad, USA).

Two- dimensional gel electrophoresis (2-DE)

OFFGEL fractionator (Agilent, USA) followed by SDS-PAGE was used as an alternative to the traditional 2-DE to separate the antigenic bands before sending for sequencing. IPG strips, Immobiline Drystrip gels with lengths of 24 cm, pH range of 3-10 (Agilent, USA) was allowed to swell in rehydrating buffer for 15 minutes. ESA was desalted by 1 ml of 5 mM Tris-HCL, pH 7.6. A volume of 800µl of desalted ESA was mixed with 3200 µl of stock solution and the final protein amount was 1mg. Subsequently 150 µl of protein sample was loaded into each well. Fractionation was initiated with a maximum current of 50 µA and maximum power of 200 mW, and typical voltages ranged from 400 to 4000 V until 64 kVh was reached after approximately 30 hours. About 100 µl of sample was collected from each well. The second dimension separation was performed by using Mini-Protein 3 cell apparatus (Bio-Rad, USA). A volume of 20 µl of each fraction was mixed with 5 µl of 5x sample buffer and directly loaded onto a 12% gel. Western blot analysis was run to verify the location of antigenic bands. The SDS-PAGE gels were then stained with Coomassie blue staining and destained with a solution of 10% acetic acid and 10% methanol.

In-gel digestion

The specific band was manually excised from the gel, digested and extracted using Agilent Protein In-gel Tryptic Digestion Kit (Agilent, USA). Briefly, the gel pieces were washed, destained at 37°C for 30 minutes, then incubated in reducing buffer at 60°C for 10 minutes. The cooled sample was then incubated with 30 µl alkylation buffer in the dark at RT for one hour followed by incubation

at 37°C with destaining buffer for 15 minutes. Washing step was performed twice, then gel pieces were shrunk by adding 50µl of acetonitrile and incubated for 15 minutes at RT, and the gel pieces were then allowed to air dry for 5 to 10 minutes. Ten µl of activated trypsin solution was added to the tube and incubated at RT for 15 minutes, followed by the addition of 25µl of digestion buffer and incubation at 30°C overnight. Digestion mixture was then removed and placed in a clean tube. Subsequently 10 µl of 1% trifluoroacetic acid (TFA) was added to the digestion mixture and incubated for 5 minutes. A final sample clean-up was performed using a Zip-Tip U-C_{18} pipette tip (Millipore, USA) before being sent for MS-MS analysis.

Mass spectrometry analysis

The selected sample was analysed by MALDI TOF-TOF 4800 at the Protein and Proteomics Centre, Faculty of Biological Sciences, National University of Singapore. GPS Explorer™ software Version 3.6 (Applied Biosystems) was used to create and search files with MASCOT search engine for peptide and protein identification. Databases from ToxoDB (http://toxodb.org/) and NCBI were used to analyse the results.

RESULTS

The *in vitro* ESA collected was concentrated 50 fold with the final protein concentration of 4mg/ml. A dilution of 1:25 was determined as the best dilution for primary antibody based on the Western blot results. For secondary antibody, the results showed that 1:8000 was the optimum dilution for monoclonal mouse anti-human IgM-HRP and 1:2000 for monoclonal mouse anti- human IgA-HRP (data not shown).

Western blot analysis using sera from Group I patients when probed with anti-human IgM-HRP and IgA- HRP revealed a consistent 10 kDa band (Figure 1 and 2). Other bands ranged from 20 to 75 kDa were also observed but they were not consistently seen in blots incubated with Group I sera. Immunoblotting performed with the control

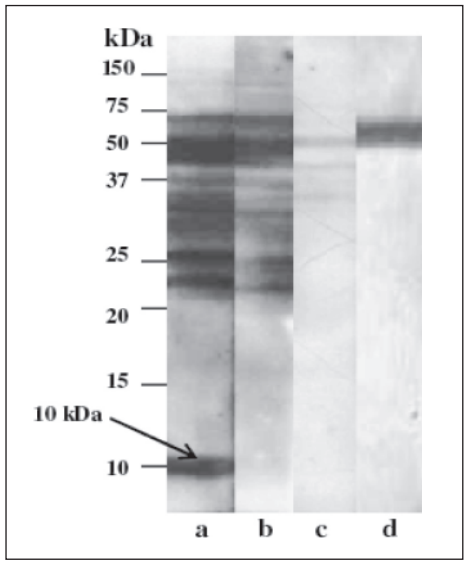

Figure 1. Representative Western blot pattern of *in vitro* grown *T. gondii* ESA recognized by different groups of serum samples when probed with anti- human IgM-HRP
a) Sample from Group I sera
b) Sample from Group II sera
c) Sample from Group III sera
d) Sample from Group IV sera

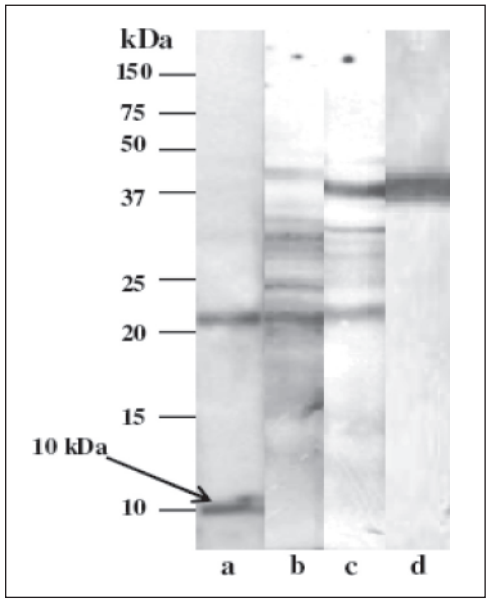

Figure 2. Representative Western blot pattern of *in vitro* grown *T. gondii* ESA recognized by different groups of serum samples when probed with anti- human IgA-HRP
a) Sample from Group I sera
b) Sample from Group II sera
c) Sample from Group III sera
d) Sample from Group IV sera

antigens, primary antibodies from Group 1 sera and the two secondary antibodies showed no reactive band, thus showing that the antigenic band originated from *T. gondii*. Table 1 shows the reactivity of this band with different groups of serum samples. In both IgM and IgA blots, the 10 kDa band gave a sensitivity of 80% among serum samples from Group I sera and specificity of 96.7% when tested with sera from Groups III and IV. Among serum samples from Group II sera, the 10 kDa band was reactive only in 2/30 and 3/30 in IgM and IgA blots respectively.

Figure 3 shows the Western blot results of the OFF-GEL fractions. Based on the locations of the 10kDa band in the fractions, the p*I* range was determined as 5.19-5.45. Table 2 shows the summary of the results from the mass-spectrometry and database analysis of the antigenic band; the two top scoring proteins were identified as ubiquitin/ribosomal protein CEP52 fusion protein and polyubiquitin.

DISCUSSION

Prognosis of toxoplasmosis depends on early diagnosis and early treatment. A variety of methods have been evaluated for the diagnosis of acute infection, classically characterized by the presence of IgM antibodies. Unfortunately, these methods which use *Toxoplasma* membrane or cytoplasmic antigens, do not always give accurate results such as false positives or false negatives, depending on the technique used. Thus there is a need to replace the crude *T. gondii* antigen preparations with a selection of well-defined antigenic proteins. One of the easiest tests for the detection of immunoglobulins is the enzyme-linked immunosorbent assay (ELISA), and many manual and automated serologic tests for the detection of *T. gondii*-specific IgG and IgM are commercially available. However, they vary in their ability to detect antibodies to *T. gondii* (Hofgartner *et al.*, 1997; Singh *et al.*, 1997; Jenum & Stray-Pedersen, 1998). Western Blot technique has also been used in order to discriminate subjects with the chronic infection from subjects with the acute form of the disease (Handman *et al.*, 1980; Kasper & Ware, 1985; Hassl *et al.*, 1987; Koch *et al.*, 1990; Moir *et al.*, 1991). In addition IgG avidity tests have recently been used to differentiate these two groups of subjects (Lefevre *et al.*, 2006; Araujo & Ferreira, 2008).

Several steps were taken in identifying antigenic bands in ESA of *in vitro* grown *T. gondii*. The first involved development of a method for producing high quality *T. gondii* ESA, while ensuring minimum contamination with soluble *T. gondii* antigens (Saadatnia *et al.*, 2010). In this study, the subsequent steps were performed which involved identification of *T. gondii* ESA antigenic component that were the most reactive with serum samples with sera of

Table 1. Summary of the reactivities of the 10 kDa antigenic band from *in vitro* ESA from blots probed with anti-human IgM-HRP and IgA- HRP

Secondary peroxidase conjugate antibodies	Group I[a]	Group II[b]	Groups III[c] and IV[d]
IgM	24/30 (80%)	2/30 (6.7%)	1/30 (3.3%) Specificity: 96.7%
IgA	24/30 (80%)	3/30 (10%)	1/30 (3.3 %) Specificity: 96.7%
IgM and/or IgA	24/30 (80%)	3/30 (10%)	1/30 (3.3 %) Specificity:96.7%

[a]Group I sera: probable acute toxoplasmosis, IgM+ IgG+ (low IgG avidity), IgM+ IgG–
[b]Group II sera: chronic toxoplasmosis, IgM– IgG+ (high IgG avidity)
[c]Group III sera: normal serum sample, IgM– IgG–
[d]Group IV sera: other parasitic infections, IgM– IgG–

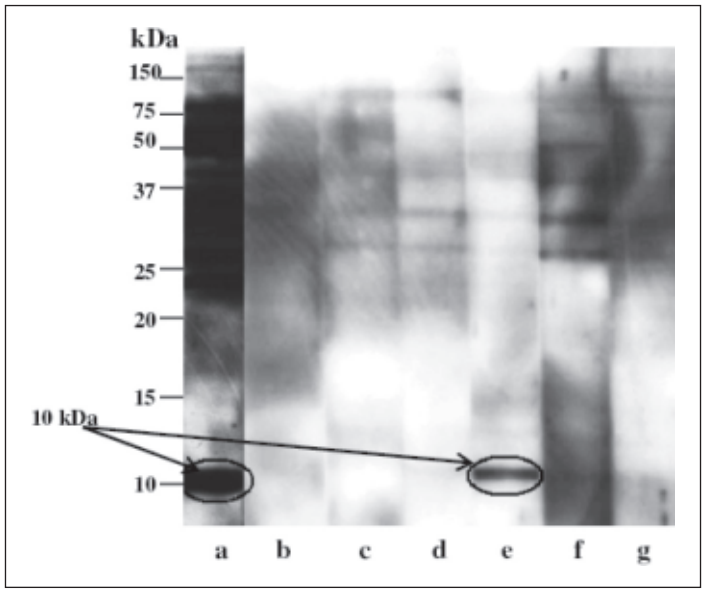

Figure 3. IgM-Western blot results on OFF GEL fractions
a) Total *in vitro* ESA
b-g) OFFGEL fractions (fractions No 1-6)

Table 2. Summary of mass spectrometric results of the ESA antigenic proteins

Antigenic proteins	Protein ID	Accession Number	p*I* range	Theoretical Mass (Da)	Search Score/ coverage
10 kDa	1. *T. gondii*_ME49 product: ubiquitin / ribosomal protein CEP52 fusion protein	TGME49_089750 NCBI: XM_002368359	9.84	14961	107 (score)
	2. *T. gondii*_ME49 product: polyubiquitin, putative	TGME49_019820			104

patients who had clinical suspicion of toxoplasmosis, and had anti-*Toxoplasma* IgM+ IgG+ or IgM+ IgG-. The sequence of the most promising antigenic component was then analysed by mass-spectrometry analysis to elucidate the amino acids and subsequently the corresponding DNA sequence.

Since the detection of IgM and IgA could suggest an active or recently acquired infection, thus the immunogenic epitopes of ESA probed by IgM and/or IgA secondary antibodies were studied. The results showed that a 10 kDa ESA protein was well-recognized by the IgM and IgA antibodies present in the sera from patients with Group I individuals with probable acute infection (80%). To determine the specificity of the antigenic bands, serum samples from three different groups, namely chronic *Toxoplasma* infection, normal/healthy individuals and other parasitic infections were used, and high specificities of 96.7% were obtained for this protein in both IgM and IgA blots. The negative results obtained with the control antigens further highlighted the specificity of the 10kDa protein.

The mass spectrometry identified two top scoring proteins for 10 kDa protein namely ubiquitin (ribosomal protein CEP52 fusion protein) and polyubiquitin. Ubiquitin is a small, regulatory protein, conserved in evolution and is present in all eukaryotic organisms (Ponder & Bogyo, 2007). The most prominent function of ubiquitin is labeling proteins for proteasomal degradation.

Polyubiquitin consists of three to five direct repeats of the ubiquitin. During periods of stress, demand for ubiquitin is augmented, results in increased levels of polyubiquitin expression (Ciechanover & Schwartz, 1994). Polyubiquitin also appears to be induced during a heat-shock response (Horrocks & Newbold, 2000; Oakley *et al.*, 2007). Ubiquitin contains several lysine residues so it can itself be ubiquitinylated and form polyubiquitin chains.

Ubiquitin exists in cells as free and also in conjugated forms. The covalent ligation of ubiquitin to various receptor proteins in eukaryotic cells regulates a number of cellular processes. Ubiquitin with ubiquitin-like proteins provide a reversible modification that control extensive range of cellular activities such as DNA repair, selective protein degradation, transcription, cellular division, endocytosis, and the processing of antigens in the immune system (Sambrook *et al.*, 1989; Jentsch, 1992; Hershko & Ciechanover, 1998). More importantly, defects in this pathway are associated with human diseases, including cancer and neurodegenerative disorders such as Parkinson's disease. By targeting disease-specific components of ubiquitin, several potential new drugs for cancer and neurodegenerative were investigated (Gonzalez *et al.*, 1996; Gantt *et al.*, 1998; Lindenthal *et al.*, 2005). Several *T. gondii* proteins related to ubiquitin (ubiquitin-like peptides or SUMO) has been identified to play a role in many diverse cellular processes. SUMO antibodies was suggested to be a potential valuable tool in toxoplasmosis diagnostics (Braun *et al.*, 2009)

Araujo & Ferreira in 2008 have reported a 30 kDa *in vitro* ESA reacted with low-avidity IgG antibodies in serum from acute toxoplasmosis. Several studies have showed utilization of recombinant *T. gondii* antigens can improve the performance of sero-diagnosis in terms of reproducibility and specificity and replace the complex native *Toxoplasma* antigens (Aubert *et al.*, 2000; Li *et al.*, 2000; Beghetto *et al.*, 2006). To extend the findings of the present study for practical use, the development of antibody detection tests can be performed using recombinant form of the 10 kDa protein or peptides that encompass the antigenic epitopes of this protein. Since ESA is secreted by the tachyzoites into the body fluid, this molecule may also be targeted in the development of antigen-detection tests for diagnosis of toxoplasmosis. This can be achieved by producing monoclonal or recombinant or polyclonal antibodies against the whole molecule or parts of the molecule that contain the antigenic epitopes. This protein demonstrated good immunogenicity, thus is probably also seen by the host soon after infection. Therefore, it may also be potentially useful for use in the development of vaccine against toxoplasmosis.

In conclusion this study successfully identified and characterized a 10 kDa protein of *in vitro* ESA of *T. gondii* which may be promising for use in diagnosis of acute toxoplasmosis.

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