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# Evaluating the Antigenic Markers of *Toxoplasma gondii* RH strain for Differential Diagnosis of Acute and Chronic Toxoplasmosis

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**ABSTRACT**

*Toxoplasmosis is one of the most prevalent parasitic infections common among humans and animals having two acute and chronic phases related to IgM and IgG, respectively. Immunoblotting is one of the serological methods with high sensitivity and specificity. Therefore, the present study aims to evaluate T. gondii RH strain antigen using the Western blot assay, their applications to distinguish between acute and chronic infections and their sensitivities and specificities. A sample of 0.02 ml of T. gondii RH strain antigen along with $10^6$ tachyzoites was injected into the peritoneal cavity of 50 white lab mice (Mus Musculus) and the peritoneal fluids of infected mice were collected after 4 days. After sonication, the protein concentration was determined using the Lowry method. Then the molecular weight of fractions was determined using SDS-PAGE electrophoresis. Seventy serum samples (30 samples of acute Toxoplasmosis, 30 chronic Toxoplasmosis and 10 serum samples of healthy individuals as controls) were investigated using the Western blot assay to identify and distinguish the antigenic markers of acute and chronic phases of the disease. Five proteins of approximately 12, 28, 41, 72, and 130 KDa were often recognized by IgG antibody present in the sera from patients with chronic toxoplasmosis. Antigens of 12, 28, and 41 KDa were recognized in all patients indicating the sensitivity and specificity of 100% for the test. Furthermore, the sensitivity and specificity of the test for the antigens of 72 and 130 KDa were 50% and 100%, respectively.*

**Keywords**: *T. gondii* Antigen, Acute toxoplasmosis, Chronic toxoplasmosis, western blot assay

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## INTRODUCTION

Toxoplasmosis is one of the most important and prevalent infectious diseases common among humans and animals. Serological evidence indicates infection throughout the world, so that more than one third of the world's population has different levels of anti-*T. gondii* antibodies; however, the prevalence of Toxoplasma infection apparently varies in different parts of the world depending on age, climatic conditions, geographical location, eating habits social customs, dispersal of definitive hosts, contact with them and immune status of different population groups (1, 2).

Toxoplasmosis is an obligate intracellular protozoan parasite that infects all kinds of live nucleated cells and has three infective stages including an active and rapidly proliferating form i.e. tachyzoites, and two resistant forms i.e. tissue cysts and oocysts. Toxoplasmosis in humans may be congenital or acquired. In congenital Toxoplasmosis, the parasite tachyzoites infect the fetus through placenta during pregnancy affects, while the acquired Toxoplasmosis occurs from the ingestion of oocysts excreted by a cat that are sporized in soil and eaten in soil, water, fruit and

vegetables or by the consumption of raw and undercooked meat of herbivores and omnivores (containing tissue cysts) (1). Acquired infection in immunocompetent persons has been often clinically asymptomatic. However, lymphadenopathy (lymph node enlargement) is the most common clinical manifestation of acquired Toxoplasmosis. Toxoplasmosis is usually crucial and dangerous for two groups of people among: Immunocompromised patients and women receiving their first exposure to acute Toxoplasmosis while pregnant (3).

Toxoplasmosis in humans consists of two phases: acute and chronic. Most complications and symptoms and the transmission of the parasite from mother to fetus occur in the acute phase. In most cases, diagnosing and distinguishing between the acute and the chronic Toxoplasmosis are made through the identification of the type and grade of specific immunoglobulins using serological methods and cytoplasmic-membrane antigens of the parasite usually associating with defects and problems. Most of these problems are created by antigens used (4).

Rapid and appropriate diagnosis of Toxoplasmosis is of great importance since timely diagnosis and appropriate treatment will significantly reduce the complications of this disease. Detecting anti-Toxoplasma antibodies in the people's serums is a common method to diagnose Toxoplasmosis. Some problems including different stability of immunoglobulins detectable by antigens used in these methods and the incidence of false-positive result due to the presence of rheumatoid factors and natural antibodies make the interpretation of the test results and the final test announcement difficult. In addition, it is observed that IgM can remain in the people's serum for long times after infection. Therefore, it cannot always be used as the indicator of recent Toxoplasmosis. In addition, most of these problems are created by antigens used in the tests designed. Therefore, diagnosing and distinguishing between the chronic and the acute forms of this disease require several serum tests performed at time intervals (5, 6). T. gondii has different strains being antigenically different. The presence of these different strains is shown using different molecular methods of RFLP, PCR, RAPD PCR and Western blot (7-9).

There are several methods to distinguish between the acute and the chronic Toxoplasmosis: Measuring anti-*T. gondii* IgM and IgA in the patient's serum using different methods (10), using IgG Avidity and ELFA Methods enjoying high sensitivity. In these methods, people placed in the suspicious group and having high antibody titer cannot be detected (11). Recombinant antigen-specific clones being costly and long term (12), the PCR method capable of distinguishing between the acute and the chronic Toxoplasmosis, yet not having suitable sensitivity (13), However, the Western blot assay could distinguish between the acute and the chronic Toxoplasmosis due to its high antigenic sensitivity and the presence of specific antigens in the acute and chronic stages. Some studies have shown that different *T. gondii* strains antigens can vary in different geographic locations. On the other hand, it is observed that the immune responses of different individuals to different *T. gondii* strains antigens are not identical and the antigenic pattern of Toxoplasmosis can vary when there are common antigens. Some studies have indicated the presence of markers detecting active Toxoplasmosis infection. Subtilisin like is a peptide similar to Subtilisin secreted by *T. gondii* Micronemes, weighed 85 kDa and having a glycosylphosphatidylinositol in its C-terminal leading to its expression on the cell surface and reacting with IgM, IgG and IgA antibodies in the serum of patients with acute Toxoplasmosis (14). A study considered P38 antigen as a diagnostic marker for the acute Toxoplasmosis (15). Other studies have shown that bradyzoites and tachyzoites existing in acute and chronic phases of the Toxoplasmosis are antigenically different. Surface antigens of tachyzoites and bradyzoites include SAG1 and SAG2 as well as SAG3 and SAG4, respectively (16). Another study showed that an antigen was detected in the serums of patients with AIDS in New York using the Western blot assay, while the above-mentioned antigen was not detected in the serums of these patients in Atlanta (17). A study conducted in Turkey showed that the standard RH strains being a non-indigenous are antigenically different with the indigenous RH strains of that region.

A study conducted in Turkey showed that the banding patterns obtained from two RH and TRH strains have been different. In this study, 14 different bands weighed 17-105 kDa were obtained for RH strain, while 4 other bands weighed 41, 52, 54 and 58 kDa were obtained for TRH strain (18). Other studies have shown that 8 bands weighed 26-105 kDa can distinguish between different strains of *T. gondii*. Bands weighed 5, 71 and 130 kDa were only in the RH strain, while 24-kDa bands and 21 and 91-kDa bands were in C56 as well as RH and T100, respectively (19). Some studies showed that pathogenic strains (RH, ENT AND MARTIN) and nonpathogenic strains (RRA, DEG and ME49) of Toxoplasma were significantly different in terms of antigenic quantity and quality, although their antigenic patterns are similar (20). Western blotting is one of the blotting techniques for the diagnosis and analysis of proteins. This method is a confirmatory. High sensitivity and specificity of immunoblotting compared to serological tests have introduced it as the superior test distinct from other methods. Particularly, it is chosen as the selected test in areas with other endemic diseases. Another reason why this method is important is the patterns of antibody responses to all investigable antigenic subunits. Validity of the Western blot assay along with ELISA test is more than 99%. Therefore, this study aims to evaluate *T. gondii* RH strain antigen using the Western blot assay, their applications to distinguish between acute and chronic infections and their sensitivities and specificities

## MATERIALS AND METHODS

### Preparation of Toxoplasma antigen

Soluble crude antigens were prepared from tachyzoites of RH strain, maintained by serial passage every 4 days in the peritoneal cavity of 30 female Balb/c mice, with average weight 25-30 mg using the method described by Yap et al. with some modifications (21). The $5x10^6$ tachyzoites were inoculated intraperitoneally in BALB/C mice 5-6 weeks old. After 4 days, 1 ml of sterile saline was injected intraperitoneally and then aspirated and examined under a light microscope. Then 0.02 ml of the fluid was inoculated into the peritoneal cavity of other mice for maintenance. The fluid was centrifuged and washed with PBS containing Tris ammonium chloride and incubated in water bath to destroy red blood cells. The suspension was centrifuged and washed and forced through a syring (needle size 27) to release the tachyzoites from peritoneum cells. Then filtrated through polycarbonate membrane(pore size 3 mm) and centrifuged and washed. After washing, tachyzoites was counted and determined viability by trypanblue stain. The antigen sonicated on ice bath 5 times for 20 seconds and centrifuged at 13000 rpm for 30 mins at 4˚C The supernatant Protein concentration was determined using the method of lowry et al. (22).

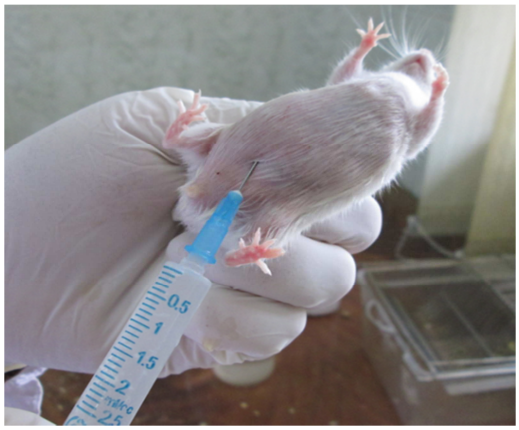

**Fig. 1. Injection of RH strain *Toxoplasma gondii* in peritoneal cavity of mice**

### Serum Sample

In this study 60 serum samples of patients with acute toxoplasmosis and chronic toxoplasmosis whose IgM and IgG antibodies were determined using commercial kits were selected to assess the sensitivity and specificity of test.

20 negative control serum samples were obtained from clinically healthy individuals.

### SDS- PAGE

*T. gondii* soluble proteins were separated by electrophoresis (Mini-protean II, Bio-Rad) on a 12% polyacrylamide gel in the presence of 10% sodium dodecyl sulphate by the method Laemmli (23).

100 µl antigen obtained (0.5 mg protein/ml) was mixed with 50 µl loading buffer 4x and boiled at 100˚ for 3 mins and centrifuged. Samples containing 0.5 mg of soluble proteins were loaded into each well. Molecular weight standards were electrophoresed on the same gel to calculate the relative molecular weights of the examined antigens. After electrophoresis, polypeptides of *T.gondii* soluble antigens stained by coomssy blue and showed bands with molecular weights ranging from 9KDa to 170 KDa.

### Western-blotting

Following SDS-PAGE, the polypeptides were transferred at 300 mA for 2 hours from the gel to a nitrocellulose membrane by the procedure described by Towbin et al. with some modifications (24). Immunoblot assay was utilized to identify immunoreactive components recognized in crude and isolated fraction of *T.gondii* using infected human sera. The nitrocellulose membranes were blocked using 5% non-fat dried milk for 24 hours in the dark at room temperature until use. Then membrane washed 3 times for 5 mins with PBS containing 0.1 % Tween 20. Human serums diluted 1:10 in PBS added and incubated for 90 min and then washed. Goat anti-Toxoplasma IgG and IgM-alkaline phosphatase conjugate diluted 1:1000 and 1:500 respectively and added to membrane for 1 hour at room temperature performed wash stages. Then substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) were added for 20 min. For stopping of reaction distilled water was used. After papering, dry revealed antigenic bands.

## RESULTS

Sera from patients with chronic and acute toxoplasmosis were tested by the presence of specific IgG and IgM against *Toxoplasma gondii*. This work helps us to demonstrate the presence of a specific protein in order to differentiate chronic and acute form of the toxoplasmosis to be used as a diagnostic marker. The fraction obtained of infected mice with SDS-PAGE showed 14 antigenic bands with ranged from 9-170 KDa.

**Western Blots**

The *Toxoplasma gondii* antigens reacted with sera from patients with chronic or acute toxoplasmosis. Thirty sera from chronic toxoplasmosis patients showed 5 bands of molecular weights ranging from 12-130 KDa. 30 sera from acute toxoplasmosis patients recognized 2 bands 10 and 41 KDa. Twenty sera from normal individuals did not show any bands (negative control).

Sera containing IgG from chronic toxoplasmosis patients showed bands 12, 28 and 41 KDa with the sensitivity and specificity of 100% and 100%, respectively and bands 72 and130 KDa with the sensitivity and specificity of 50% and 100%, respectively.

Sera containing IgM from acute toxoplasmosis patients showed bands 10 and 41 KDa with the sensitivity and specificity of 100% and 100%, respectively. The 41 KDa band recognized by antibodies in both chronic and acute patients but could not be considered as a diagnostic markers.

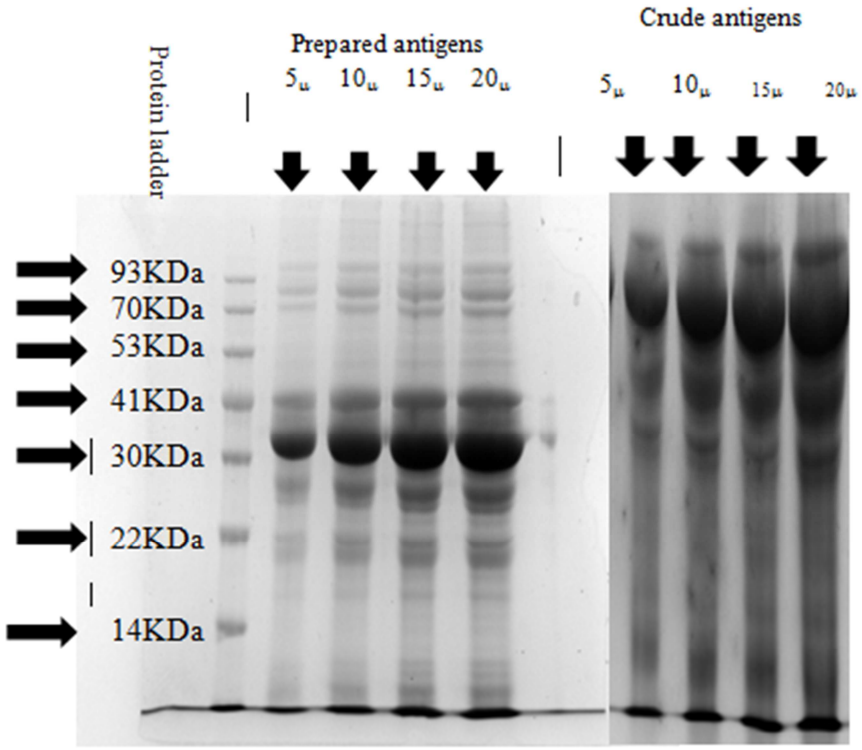


**Fig. 2. Results of electrophoresis peritoneal fluid of infected mice with *Toxoplasma gondii* of RH strain on 12% gel SDS-PAGE**

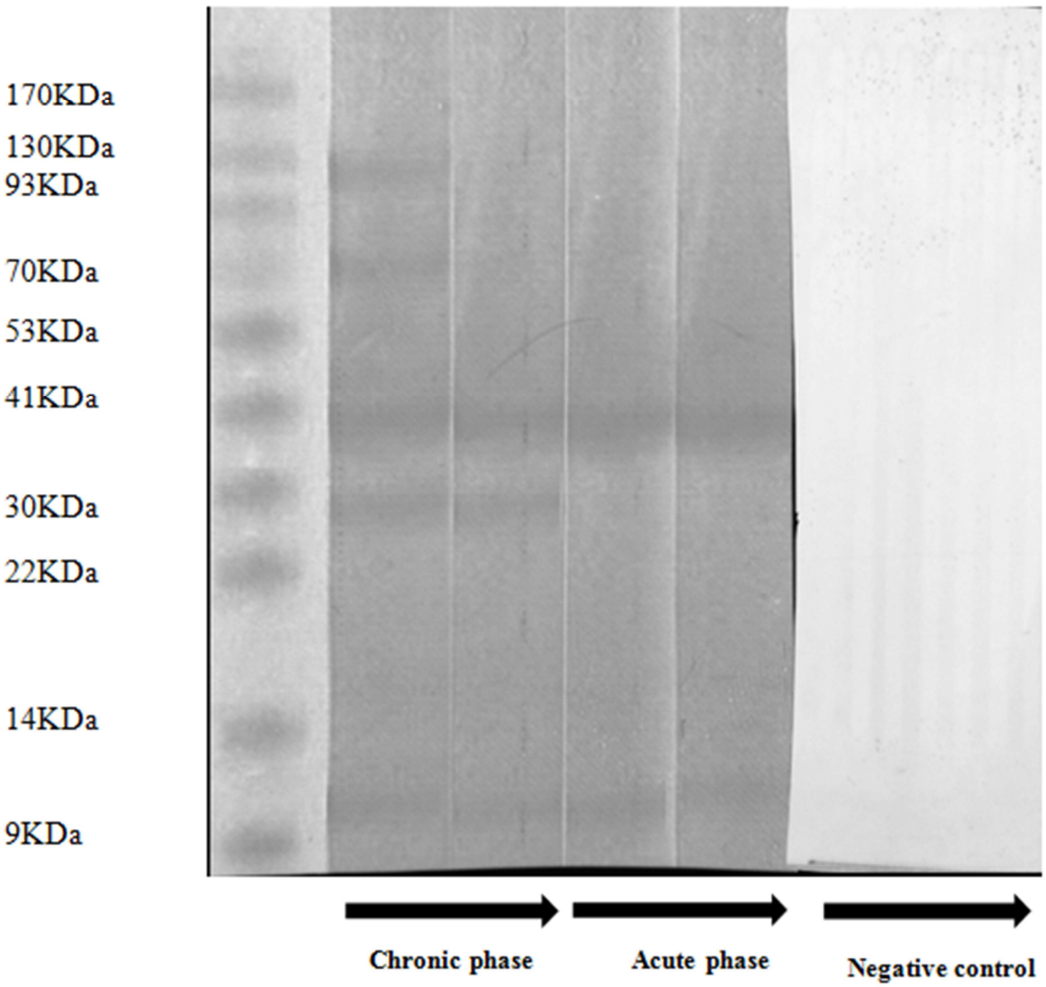


**Fig. 3. Results of immunoblotting of the *Toxoplasma gondii* RH strain with different sera samples**

## DISCUSSION

Crude *Toxoplasma gondii* tachyzoites antigens represents as a raw material used to prepare fractions to be employed in western-blotting test for detecting antigenic markers in chronic and acute toxoplasmosis. The actual antigenic markers of the parasite involved in the interaction with *T. gondii* antibodies need further investigations, where antigens of Toxoplasma are complex and diverse. The interactions between *T.gondii* antigens and IgG and IgM antibodies depend on the wide range of hosts susceptible to *T. gondii* (25). In the present study, five proteins of approximately 12, 28, 41, 72, and 130 KDa were often recognized by IgG antibody present in the sera from patients with chronic toxoplasmosis. Antigens of 12, 28, and 41 KDa were recognized in 30 of 30 patients therefore the sensitivity and specificity of test were detected 100% and 100%, respectively, and antigens of 72 and 130 KDa were recognized in 15 of 30 patients with chronic infection, therefore the sensitivity and specificity of test were evaluated 50% and 100%, respectively. Two proteins with 10 and 41 KDa were recognized by IgM antibody present in the sera from patients with acute toxoplasmosis. Antigens of 10 and 41 KDa were recognized in 30 of 30 patients; therefore the sensitivity and specificity of test were evaluated 100% and 100%, respectively. Antigens of 12 and 10 KDa could be recognized in chronic and acute toxoplasmosis, respectively. Susanto et al. (2001) were observed bands of molecular weight 41, 72, 90, 87, 82, 24, 26 and 36 KDa in chronic and acute toxoplasmosis (26). Bour et al. (2006) were detected bands of molecular weight 160, 120, 80, 30 and 35 KDa in chronic toxoplasmosis (27). Attallah et al. (2006) separated band of molecular weight 36 KDa in acute toxoplasmosis (28). Yaman et al. (2011) were identified in RH and Ankara strain bands of molecular weight 60-70 KDa and TS-4 strain 60 KDa (29). Hruzik et al. (2011) were detected SUB1 like with molecular weight 85 KDa in acute toxoplasmosis (14). Hassan et al. (2012) were identified bands of molecular weight 23 and 65 KDa in LA strain and 65 KDa in LAb strain (30). Hassanain et al. (2013) were separated band of 116 KDa in chronic and acute toxoplasmosis (31). Additional detailed studies using the western blotting in characterization of different bands in chronic and acute toxoplasmosis may enhance the understanding of the host/parasite relationship and give some insight into the pathogenicity and

immunogenicity of this parasite. Further research is needed to confirm if the proteins of 12 and 10 KDa can be considered as diagnostic markers for the chronic and acute stages of toxoplasmosis.

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