PREPARATION OF *TOXOPLASMA GONDII* RH STRAIN ANTIGEN, ANTIGEN ANALYSIS AND ANTIGEN DETECTION IN SERA: A REVIEW

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Abstract. The prevalence of antibodies detected by serology against *Toxoplasma gondii* in Indonesia is quite high. Therefore further research concerning the antigen used is important to improve the quality of the assay being used with lower cost. An attempt to prepare *T. gondii* strain RH antigen followed by using it in the ELISA procedure for detecting IgG showed that there was no significant difference between the local ELISA and Toxonostika quantitatively and qualitatively.

Analysis of the antigen was done by Western blot, using sera collected from 30 clinically suspected toxoplasmosis cases which contained IgG only (titer ranging from 1:1,600-⩾ 1:3,200); from 9 asymptomatic healthy person, from 5 cases consisted of 1 case of lymphadenitis (IgM titer ⩾ 1: 3200 and IgG titer 1:800) and 4 cases of visual disturbances which had IgM with titer ranging from 1:800 to ⩾ 1: 3,200 and IgG with titer ranging from 1:800 to ⩾ 1: 3,200. It was shown that antigen components of 90, 87, 82, 72, 41, 26 and ⩽ 6 kDa reacted to all sera containing IgG except sera containing both IgG and IgM. Especially bands of 41 and 26 kDa showed strong reaction with all sera containing IgG, except 2 sera which contained both IgG (titer 1:800) and IgM (titer 1:800 and 1:3,200). These sera collected from 2 left eye vision disturbance cases were not reactive to all antigen components. Strong reactions against bands of 41, 26 and ⩽ 6 kDa were also shown in sera which contained only IgG collected from 9 healthy persons without any toxoplasmosis symptoms whereas bands of 90, 87, 82 and 72 kDa all showed moderate strong reaction. Contrasting to sera containing only IgG, of 5 sera containing both IgG and IgM 3 of them showed only reactions against bands of 41, 26 and ⩽ 6 kDa which were strong. It seemed that almost all sera containing IgG gave reaction to 90, 87, 82, 72, 41, 26 and ⩽ 6 kDa, however different pattern of reaction might occur, probably depending on the nature of infection as more antigen components would be recognized by sera containing IgG alone rather than sera containing both IgM in large quantity and IgG.

In another study, an attempt to detect *T. gondii* antigen in 60 samples was done by using ELISA, and it was shown that circulating antigen was found in 27 (90%) from 30 samples which contained both IgG and IgM, whereas only 2 (66%) from 30 samples which contained only IgG showed positive results. Therefore, antigen detection can be used to identify the acute phase of infection.

INTRODUCTION

Toxoplasma gondii is an intracellular protozoon which can infect many mammalian species. In human the parasite can cause among other things congenital anomalies in babies, 2-7% per baby birth (Feldman and Remington, 1987; Wilson, 1990). In a study in pregnant women in Dr Cipto Mangunkusumo Hospital in Jakarta the prevalence was 14.3% and 67.8% in 50 cases of abortion, whereas in patients with a history of one or more abortions or stillbirths the prevalence was 21.5% and 22.8% respectively. In adults and children with chorioretinitis the prevalence of antibody was 60% whereas it was 17% in patients with other eye lesions (Gandahusada, 1991).

In adult and babies the infection can be asymptomatic, therefore laboratory examinations which include direct examination, parasite isolation and serological methods is very important. Direct examination to find the parasite is difficult, and it needs special staining. Parasite isolation will take a long time and it cannot differentiate between acute and chronic infection. The difficulty to assess the nature of infection can be overcomed by serological examination, which can be done in asymptomatic suspected cases as well as in suspected infected tissues.

Various serological methods are used to diagnose toxoplasmosis *ie* enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFA), IgM immunosorbent agglutination assay (IgM ISAGA), hemagglutination, complement fixation reaction and Sabin-Feldman test. However, the hemagglutination, complement fixation reaction and Sabin-Feldman test can only detect IgG, whereas IgM ISAGA is used only to detect IgM. Both ELISA and IFA can detect either IgG or IgM. Quantitatively, it was shown that ELISA was more sensitive and more specific for serodiagnosis of toxoplasmosis, it had a positive correlation with the IFA (Wall *et al*, 1988), dye test and the hemagglutination test (Voller *et al*, 1976). Other studies also showed that the ELISA was more sensitive as the titer could reach 20-40 fold higher than IFA and

200-1,000 fold higher than complement fixation reaction (van Loon and van der Veen, 1980). Therefore, in comparison to IFA, ELISA was more objective and more frequently used because it was easier and more practical, especially for large amount of samples (Voller *et al*, 1976). It was also shown that for detecting IgG in toxoplasmosis the ELISA did not show any cross reaction with other parasites *eg Wuchereria bancrofti*, *Loa-Loa*, *Onchocerca volvulus*, *Schistosoma japonicum*, *Echinococcus granulosus* and *Entamoeba histolytica* (Dahl and Johnson, 1984). Another kind of ELISA: the double sandwich IgM ELISA was initiated by attaching a specific antibody against IgM on the wells surface of the plate. This procedure could prevent false positive results in sera containing rheumatoid factor and it could also prevent false negative results from excess of IgG (Payne *et al*, 1982). In ELISA the antigen used is very important, therefore further research concerning the antigen used is important to improve the quality of the assay being used. Until present the Toxonostika kit used for ELISA has to be imported. An attempt to prepare *T. gondii* strain RH antigen that can be used in local ELISA which will be less expensive and then comparing the result with the ELISA Toxonostika had been made recently. This paper reports the preparation of *T. gondii* RH strain antigen, antigen analysis and its detection in sera.

PREPARATION OF *TOXOPLASMA GONDII* RH STRAIN ANTIGEN

The tachyzoites of *T. gondii* RH strain were inoculated intraperitoneally in 5-6 weeks old albino mice. Every 4 days, 1 ml of saline was injected intraperitoneally and then aspirated. The intraperitoneal fluid was examined under a light microscope and if the tachyzoites were > 10 per microscopic field then 0.02 ml of the fluid was inoculated into the peritoneal cavity of 3 other mice for maintenance.

The preparation of antigen was done according to the technique described by Yap (1983). Briefly, peritoneal fluid was collected from peritoneal cavity of 25 mice which were inoculated with 0.02 ml fluid containing tachyzoites 4 days before. The fluid was centrifuged and a suspension of the sediment in 0.9% sodium chloride containing Tris ammonium chloride was incubated in shaking water bath to destroy red blood cells. The suspension was centrifuged twice and washed with 0.9% sodium chloride four times and the final suspension was forced through a disposable syringe (25 ml, needle size 27) to release the tachyzoites from peritoneum cells. After being filtrated through polycarbonate membrane (pore size 3 mm), centrifuged and then washed five times, the tachyzoites were counted and suspended in 0.9% sodium chloride before storing at -20^{o}C. If required, the antigen would be sonicated in ice bath and the "soluble antigen" was stored in -20^{o}C. This antigen was used in the local ELISA for IgG and the result was compared qualitatively and quantitatively to ELISA Toxonostika. The ELISA was performed on 163 sera and it was shown that there were 8 sera which showed positive results by ELISA Toxonostika but negative by the local ELISA, whereas 5 sera were negative with ELISA Toxonostika but positive by the local ELISA. Statistically, it was shown that there was no significant difference of qualitative results between these two assays ($p>0.05$) and there was a strong correlation between IgG titer of both assays (Rahmad, 1994). These results were very encouraging, therefore further attempt for developing a local ELISA for IgM will be made in the future.

ANTIGEN ANALYSIS

Antigen analysis was done by Western blot in which the antigen components were separated by SDS-PAGE and identified by antibodies. After electrophoresis the antigen components were transferred onto nitrocellulose membrane and cut into stripes. The stripes were incubated with sera from patients, hence reacted with enzyme labeled antihuman immunoglobulin and substrate to form bands. The amount of the bands and their thickness depends on the amount of antibodies which reacted with the antigen. By this method molecular weight of immunogenic molecules could be assessed (Hames, 1990; Rollag and Edelstein, 1992). Although a protein could be separated into its components according to their molecular weights in SDS-PAGE, they could not react with antibody because SDS inhibited the antigenic function of protein, and antibody could not enter into the polyacrylamide gel matrix. Therefore the protein antigen should be transferred from the polyacrylamide gel to the surface of nitrocellulose membrane by electrotransfer. The antigen-antibody complex could be detected by colorimetry or chemiluminence after labeled with human globulin conjugated with enzyme or radioactive materials (Maizels and Robertson, 1988).

In a study, the tachyzoite suspensions (concentration $1x10^{9}$ organisms/ml) were sonicated to yield 1.8-2 mg protein/ml (Setyo, 1998). The sera used were categorized into 3 groups:

1. Sera containing IgG only with titer of $\geqslant$ 1:3,200 consisted of 30 clinically suspected patients (25 cases of eye lesions, 2 cases of lymphadenitis and

Table 1
Distribution of sera containing only IgG collected from 30 suspected toxoplasmosis cases.

No. of sera	Sex	Age (yrs)	Symptoms
01	F	-	Abortion (7 months)
02	M	42	Toxoplasmic tumor
03	F	19	Lymphadenitis
04	M	26	Chorioretinitis OD
05	M	52	Chorioretinitis OD
06	F	22	Cicatrix regio macula OD
07	F	12	Chorioretinitis OS (recidif)
08	F	55	Chorioretinitis centralis OD
09	F	26	Chorioretinitis sanata
10	F	54	Cicatrix regio macula OD
11	M	-	Chorioretinitis
12	M	6	Chorioretinitis
13	F	21	Cicatrix regio macula centralis
14	F	12	Chorioretinitis centralis OS
15	M	22	Chorioretinitis centralis
16	F	17	Chorioretinitis centralis
17	F	20	Chorioretinitis OS
18	M	24	Lymphadenopathy coli
19	F	23	Choroiditis OS
20	F	27	Chorioretinitis
21	F	13	Chorioretinitis sanata
22	M	37	Retinochoroiditis
23	M	21	Choroiditis OS
24	F	18	Chorioretinitis OS
25	M	40	Choroiditis OS
26	F	27	Abortion (2 X)
27	M	6 1/2	Choroiditis OS
28	F	14	Cicatrix regio macula OD
29	M	31	Choroiditis
30	M	31	Active choroiditis

Quoted from Setyo (1998).

lymphadenopathy, 1 case each of tumor, abortion and stillbirth) (Table 1) and 9 sera collected from asymptomatic healthy persons.

2. Five sera containing IgM and IgG antibodies against *T. gondii* which consisted of:

a. serum with IgM titer of *f* 1: 3,200, IgG 1:800 collected from 1 case of lymphadenitis,

b. serum with IgM titer of 1:3,200 and IgG titer of 1:800; with IgM titer of 1:1,600 and IgG titer of 1:100; with IgM titer of 1:200 and IgG titer of 1:3,200; and with IgM titer of 1:800 and IgG titer of 1:800, all 4 sera were collected from cases with left eye disfunction. No serum with IgM positive and IgG negative was found in this study.

3. Ten ELISA negative sera collected from healthy persons as negative control.

Briefly, the SDS-PAGE was done by using Mini V8-110 vertical gel electrophoresis from Gibco BRL. Electrophoresis of *T.gondii* antigen was done by using 15% stabgel acrylamide (Mini V8-10 vertical Gel Electrophoresis System. Instruction Manual cat Serie 1078 BRL Life Technologies Inc) for 45 minutes. As indicator the bromophenol blue was used, and after SDS-PAGE the protein antigen components were transferred onto nitrocellulose membrane. Then the membrane was cut into stripes. Stripes containing standard protein were dyed with Indian ink whereas stripes containing protein antigen were immunologically detected *ie* reacted with sera (dilution 175:1,000), horse radish peroxydase conjugated rabbit antihuman immunoglobulin, and 4-chloro-1-naphthol containing 0.01% H_2O_2. The bands formed by reaction of antigen components with IgG originated from various molecular weight of components, ranged from 90 kDa to ⩽6 kDa. There were at least 19 bands, however not all sera containing IgG have the same pattern of reaction, only components of 90, 87, 82, 72, 41, 26 and ⩽6 kDa reacted with all sera containing only IgG *eg* sera collected from 30 clinically suspected cases reacted against 90, 87, 82, 72 and ⩽6 kDa with different reaction intensity. However components of 90, 87, 82 and 72 kDa reacted uniformly moderate strong with sera collected from 9 asymptomatic cases. These two groups also reacted uniformly strong against components of 41 and 26 kDa with the additional uniformly strong reaction against component of ⩽6 kDa in the asymptomatic group, unlike the clinically suspected cases (Table 2,3). Contrastingly only components of 41, 26 and ⩽6 kDa reacted with sera containing both IgG and IgM (Table 4). It seemed that more antigen components were recognized by sera containing IgG only than sera containing both IgG and IgM, probably because of the nature of infection in which IgG would be produced later and in a longer period than IgM (in chronic infection) (Partanen *et al*, 1984). In sera containing both IgG and IgM positive reactions which were strong were shown against antigen components of 41, 26 and ⩽6 kDa. This phenomenon was shown by 3 of 5 sera used, in which the IgM reciprocal titer was at least four fold IgG reciprocal titer (Table 4, serum No. 1, 2 and 3). However equal titer of IgG and IgM (1:800) or IgG reciprocal titer sixteen fold to IgM reciprocal titer showed negative results (Table 4, serum No. 4 and 5). Could these differences reflect the nature of infection? Further research using more samples should

Table 2
Pattern of reaction of sera collected from suspected toxoplasmosis cases containing only IgG against antigen components of *T. gondii* RH strain shown by Western blot.

No.of sera	Molecular weight of antigen components of *T. gondii* strain RH (kDa)																		
	90	87	82	72	70	68	57	50	48	41	39	37	33	26	23	22	21	15	9	⩽6
01	+	+	+	+	0	±	±	±	±	++	0	0	0	++	0	0	0	+	0	+
02	+	+	+	+	0	+	+	0	+	++	0	0	0	++	0	0	0	±	0	+
03	+	+	+	+	+	0	±	0	0	++	0	0	0	++	0	0	0	±	0	+
04	+	+	+	+	0	0	±	0	0	++	0	0	0	++	0	0	0	±	0	±
05	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	±	0	±
06	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	+
07	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	+	0	+
08	+	+	+	±	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	±
09	±	±	±	++	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	±
10	±	±	±	±	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	+
11	±	±	±	±	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	±
12	+	+	+	+	0	+	+	+	+	++	0	0	0	++	±	±	+	+	+	++
13	+	+	+	+	±	±	+	+	0	++	0	0	0	++	0	0	+	±	±	++
14	+	+	+	+	±	0	+	0	0	++	0	0	0	++	0	0	±	±	±	++
15	+	+	+	+	0	0	+	0	0	++	0	0	++	++	0	0	±	±	±	++
16	+	+	+	+	0	0	+	0	0	++	0	0	0	++	±	±	±	0	+	±
17	+	+	+	+	0	±	+	0	+	++	0	0	0	++	0	0	±	0	0	±
18	+	+	+	+	0	0	+	0	0	++	0	0	0	++	0	0	+	0	0	+
19	+	+	+	+	0	+	+	0	0	++	0	0	0	++	0	0	0	0	±	±
20	+	+	+	+	0	0	+	0	0	++	0	0	0	++	0	0	0	0	+	++
21	+	+	+	+	0	±	+	0	0	++	0	0	0	++	0	0	0	0	0	++
22	+	+	+	+	0	0	±	0	0	++	0	0	0	++	0	0	0	0	0	±
23	+	+	+	+	+	0	±	±	0	++	+	+	+	++	0	0	0	0	0	++
24	+	+	+	+	±	±	+	±	0	++	0	0	0	++	0	0	0	0	0	+
25	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++
26	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	±
27	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	+
28	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	±
29	+	+	+	+	0	0	+	0	0	++	0	0	0	++	0	0	0	0	0	++
30	+	+	+	+	0	0	0	0	0	++	0	0	+	++	+	0	0	0	0	++

Quoted from Setyo (1998).

0 : no reaction; ± : weak reaction; + : moderately strong reaction; ++ : strong reaction

therefore be conducted in the future.

ANTIGEN DETECTION IN SERA

The presence of *T. gondii* infection can be determined in many ways. Inoculation technique into mouse was very specific and sensitive but it would take a long time (approximately 6 weeks), a more rapid way was to isolate the organism on tissue culture (Foulon *et al*, 1990). Other technique, the polymerase chain reaction (PCR) had been used extensively to detect various diseases (Grover *et al*, 1990).This technique could detect the DNA of one parasite per sample in only one day however this assay was very expensive (Grover *et al*, 1990). To determine the nature of infection of *T. gondii* is very important because treatment of acute infection in pregnant mothers will be very beneficial to minimize congenital disorders in the fetus. Usually acute infection could be diagnosed by serology; *ie* by detecting IgM and for past infection by detecting IgG (Hohlfeld *et al*, 1989). However, detecting antibodies in immunosuppressed patients is not adequate since the immune response is diminished.

Table 3
Pattern of reaction of asymptomatic human sera containing only IgG against antigen components of *T. gondii* RH strain shown by Western blot.

No.of sera	Molecular weight of antigen components of *T. gondii* strain RH (kDa)																		
	90	87	82	72	70	68	57	50	48	41	39	37	33	26	23	22	21	15	9	⩽6
01	+	+	+	+	+	0	+	+	0	++	0	0	0	++	±	0	0	+	0	++
02	+	+	+	+	+	±	+	+	0	++	0	0	0	++	+	0	0	+	±	++
03	+	+	+	+	+	0	+	0	0	++	0	0	0	++	±	0	0	+	0	++
04	+	+	+	+	+	0	+	0	0	++	0	0	0	++	±	0	0	0	0	++
05	+	+	+	+	+	0	+	+	0	++	0	0	0	++	±	0	0	+	±	++
06	+	+	+	+	+	0	0	0	0	++	0	0	0	++	0	0	0	±	0	++
07	+	+	+	+	+	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++
08	+	+	+	+	+	0	0	0	0	++	0	0	0	++	0	0	0	±	0	++
09	+	+	+	+	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++

Quoted from Setyo (1998)
0 : no reaction; ± : weak reaction; + : moderately strong reaction; ++ : strong reaction

Table 4
Pattern of reaction of sera containing IgM and IgG against antigen components of *T. gondii* RH strain shown by Western blot.

No.of sera	Molecular weight of antigen components of *T. gondii* strain RH (kDa)																		
	90	87	82	72	70	68	57	50	48	41	39	37	33	26	23	22	21	15	9	⩽6
01	0	0	0	0	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++
02	0	0	0	0	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++
03	0	0	0	0	0	0	0	0	0	++	0	0	0	++	0	0	0	0	0	++
04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Quoted from Setyo (1998)
0 : no reaction; ± : weak reaction; + : moderately strong reaction; ++ : strong reaction

Therefore it was very helpful to diagnose acute toxoplasmosis by detecting circulating specific antigen (Acebes *et al*, 1994).

Invasion of *T. gondii* in human macrophages was initiated by the attachment of the anterior portion of the organism on the macrophage cell membrane (Wyler, 1990). In this attachment process a penetration enhancing factor (PEF) was secreted. Another protein would be excreted when the organism actively invade the macrophage as metabolite, and the presence of these proteins as circulating antigen indicated an active infection (Cazabone *et al*, 1994). Other studies showed that these circulating antigen could be detected only for a short period *ie* in the active phase, therefore antigen detection could determine the presence of acute infection (Hafid *et al*, 1995). The tachyzoites also contained various proteins, one of it was an enzyme, the nucleotide triphosphate hydrolase (NTPase). The NTPase weighted 250 kDa, and the concentration was found quite high in acute toxoplasmosis cases. It was shown that NTPase concentration correlated with the severity of disease (McLeod *et al*, 1991).

A study by using ELISA had been done to detect *T. gondii* antigen in 30 positive IgM, positive IgG samples and 30 negative IgM, positive IgG samples (Sukri, 1998). The result was regarded positive if the sample absorbance was 2 standard deviation more than the absorbance of negative control. It was shown that antigen was found in 27 (90%) samples from 30 samples with positive IgM and positive IgG but only

in 2 (6.6%) samples from 30 samples with negative IgM and positive IgG, and these 2 samples had IgG titer of 1:3,200. Not all samples containing positive IgM and positive IgG were positive for antigen, probably because circulating antigen could be detected only in a short time (van Knapen, 1977), whereas the IgM could be detected for a longer time in sera (Sukri, 1998). In mice infected with *T.gondii*, the antigen could be detected 48 hours after infection, and the concentration peak would be reached on the tenth day after infection (Acebes *et al*, 1994). In this study antigen was found only in samples with negative IgM and high IgG titer (1: 3,200), probably because these samples might be taken from reactivated infection cases, in which IgM was seldom detected. It was concluded that antigen detection could be used to detect the early stage of infection. Detecting circulating antigen is also a rapid and sensitive way to diagnose acute phase, however further studies for diagnosing reactivation process should be done in the future.

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