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Toxoplasma gondii Identification in Mother's Blood and Fetal Tissue with Nested PCR

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Abstrak

Toxoplasma gondii identification in mother's blood and fetal tissue with nested PCR
Objective: To examine the correlation between Toxoplasma gondii infection with spontaneous abortion on pregnant women based on nested PCR result from mother's blood and fetal tissue.

Methods: A prospective clinical diagnostic study using nested PCR performed on 30 cases of pregnant women with spontaneous abortion fulfilling the inclusion criteria, latex agglutination test (+) and exclusion criteria, latex agglutination test (-). Mother's blood and fetal tissues samples, which gave positive result in serologic test, were analyzed with nested PCR using18S-rDNA gene primers.

Results: Five of 30 mother's blood samples (16.7%) and 9 of 30 fetal tissue samples (30%) gave positive PCR results. According to Fisher's Exact test, PCR detected the presence of Toxoplasma gondii in significant value (P < .001). Conclusion: There is a strong correlation between Toxoplasma gondii infection with spontaneous abortion (P < 001).

Keywords: *nested PCR – Toxoplasma gondii infection – spontaneous abortion*

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infect an extremely wide host range, from birds to mammals, including humans. This parasite can survive in all nucleated cells, including blood cells in acute stage, forms a specific vacuole that protect the parasite from host cell immune system. In the chronic stage, the parasite can form a cyst in the central nervous system, skeletal muscle and eye tissue and can exist for the lifetime of its host. The cysts can rupture and release highly invasive trophozoite, which may cause a recurrent infection and potentially fatal if the host is in a state of immune deficiency.[1,2,3]

Toxoplasma gondii infection is often asymptomatic in healthy individuals, but a primary *Toxoplasma gondii* infection in pregnant women may cause a range of abnormalities including abortion, fetal death and congenital defect, depending on gestation age when infection occurs. The fetus is infected by Toxoplasma gondii through placental circulation. The cyst can form in placental tissue and fetal brain. When the fetus is infected at first trimester, abortion can occur. In Norway between 1992 to 1994, 10.9% women were infected before pregnancy and 0.17% were infected during pregnancy. In Indonesia, toxoplasmosis prevalence is 14% and it is still high in pregnant women, about 5.5-84%. The high score of toxo-plasmosis in pregnant women, especially asymptomatic or silent infection, will limit and cause difficulty in the diagnosis process so the abnormality or mortality of the fetus will be increased.[4,5,6,7]

Current diagnosis of Toxoplasma gondii is based on parasite isolation and serological assay. *Toxoplasma gondii* can be isolated by mice inoculation or tissue cul-ture but this technique need longer time, about 3-6 weeks. Serological test can overcome this problem and detect *Toxoplasma gondii* anti-body. Positive serologic result was determined by showing a seroconversion of immunoglobulin G antibodies for primary infection and detection of specific immunoglobulin M. This method is also time-consuming and complicated by the presence of crossreactive antibodies, and influenced by immunology condition, especially in immunosuppression or immunodeficiency patients.[8,9,10,11,12,13]

Based on this fact, PCR becomes a very important diagnostic tool because it has a very high sensitivity and specificity in detecting *Toxoplasma gondii* infection, both in acute and chronic stage. One of the PCR methods which has very high sensitivity and accuracy is a nested PCR using primer which is very conserved and species-specific[14], like 18S-rDNA primers used in this study. The PCR is performed both on mother's blood and aborted tissue and the result can be performed in a few hours.[15-22]

Materials And Methods

Patients' characteristic and selection. (i) Mothers. All pregnant women with spon-taneous abortion within 20 weeks of gestation from October 2002 to March 2003 were identified and characterized by age, gestational age, spontaneous abortion history and parity. They were offered serological test (agglutination test) and suggested to perform PCR examination both on mother's blood and fetal tissue if serological test gives positive result. After the informed consent was granted, 5 cc venous blood was drawn and centrifuged to separate its serum from blood, then the serum was serologically tested. Thirty preg-nant women with abortion and whose gave positive result in serologically test were included in this study. The remains of blood samples were preserved at -20^{o}C for subsequent DNA isolation step. **(ii) Fetal tissue.** Five cc fetal tissues from all serologic positive mothers were taken and preserved at -20^{o}C for subsequent DNA isolation step.[22,23,24]

Selection of Primer. Primer used for this nested PCR will amplify a DNA sequence from 18S-rDNA gene. The primer was used in Keio University, Japan and pro-ven to give positive result in HIV-positive individuals with encephalitis toxoplasmosis. The first primer pair produces a 311-bp DNA segment from base 48 to 359 and the second primer pair produces a 290-bp DNA segment from base 58 to 348. The first primer pair used are 5'-CCATGCATGTCTAAGTATAA GC and 5'-GTTACCCGTCACTG CCAC. The second primer used are 5'-CTAAGTATAAGCTTTTATACG GC and 5'-TGCCACGGTAGTCC AATAC.

Preparation of DNA tem-plate for PCR. DNA templates we-re prepared from mother's blood and fetal tissue. Fifty µl of each sample was putt into 2.5 ml a Eppendorf tube and 100 µl Triton X-100 was added into the tube. Then the tube was boiled for 5 minutes and stored at -20^{o}C until used. This technique was used in Keio University Japan and has proven to be successful in PCR process.

Amplification protocol. The reagents used for PCR amplification were given from Keio University Japan. PCR mix-tures were prepared for 50 µl react-ion volume as following: distilled water 33.25 µl, 10xPCR buffer 5 µl, 2mM dNTPs 5µl, 25 mM $MgCl_2$ 4µl, each primer 0.5µl, Taq poly-merase 0.5µl and DNA template 1.5 µl. DNA thermal cycler (Perkin-Elmer) was programmed for 40 cycles of amplification, both for first and second round PCR. Parameters for the first round PCR cycle consisted of 5 min at 95^{o}C (initial denaturation), 30 sec at 94^{o}C (denaturation), 1 min at 64^{o}C (primer annealing), 2 min at 72^{o}C (polymerization) and 5 min at 72^{o}C

(extended polymerization). Parameters for the second round PCR consisted of 5 min at 95°C (initial denaturation), 30 sec at 94°C (denaturation), 1 min at 60°C (primer annealing), 2 min at 72°C (polymerization) and 5 min at 72°C (extended polymerization). An aliquot of the reaction mixture was electrophoresis analyzed by 2% agarose gel stained by 2 µl ethidium bromide and visualized under UV light. A UV Camera was used to take its photograph using a 559 Polaroid film.

Result

Thirty pregnant women with spontaneous abortion within 20 weeks of gestation, characterized by age, gestational age and spontaneous abortion history, who gave positive result in serological test were submitted to nested PCR. Nested PCR showed 5 positive results from mother's blood and 9 positive results from fetal tissue. (Figure 1, 2 and 3)

Based on PCR result, women at age 20-29 had the largest positive cases with 2 cases (6.67%) from mother's blood and 3 cases (10%) from fetal tissue, including a positive case both on mother's blood and its fetal tissue. Women at age below 20 showed 4 positive cases, each 2 cases from mother's blood and their fetal tissue (6.67%). In women aged 30-39, there were also 4 positive cases, 1 case (3.33%) from mother's blood and 3 cases (10%) from fetal tissue. There was only 1 positive case from fetal tissue on women aged over 40. (Table 2)

On gestational age, the largest positive cases were found at $\geq$ 8-12 weeks pregnancy, with 4 cases (13.33%) from mother's blood and 8 cases (26.67%) from fetal tissue. Four positive cases from mother's blood consisted of each 2 cases at 8-10 and 10-12 weeks pregnancy, all of them showed positives result in their fetal tissue samples. Eight positive cases from fetal tissue consisted of each 4 cases at 10-12 and 8-10 weeks pregnancy. At gestational age below 8 weeks, we found 1 positive result (3.33%), both on mother's blood and fetal tissue, but there are no positive results at $\geq$ 12-16 and $\geq$ 16-20 weeks pregnancy. (Table 3)

According to spontaneous abortion history, women with first spontaneous abortion history gave the largest positive results with 5 positive cases (16.67%) from mother's blood and 7 positive cases (23.33%) from fetal tissue, 5 positive results occurred both on mother's blood and their fetal tissues. Each followed by 1 posi-tive case from fetal tissue for women with 2-3 times and more of 3 times spontaneous abortion history. (Table 4)

All data were analyzed with Fischer's Exact Test and showed significant relationship ($P < .001$, 95% CI) between fetal tissue and mother's blood examination by nested PCR.

Discussion

This study has some advantages. First, it describes the characterization of women with spontaneous abortion, which were infected by *Toxoplasma gondii* and the prevalence of toxoplasmosis at Malalayang General Hospital (RSUP) Manado, North Sulawesi, Indonesia. Second, it shows the significant advantage of nested PCR to detect *Toxoplasma gondii* in mother's blood and fetal tissue and estimate the stage of *Toxoplasma gondii*, whether it is in acute or chronic form. Third, the study also shows the correlation between fetal tissue and mother's blood PCR result. Beside some ad-vantages above, there is also a possibility that patients with positive serological result had no full protection because they still had a chance to get toxoplasmosis.

Based on the result, we can describe the pattern of *Toxoplasma gondii* infection in women with spontaneous abortion at Manado, who had a serological positive result, according to age, gestational age, spontaneous abortion history and parity.

The result showed that from 30 pregnant women with spontaneous abortion, women at age 20-29 had the higher risk for toxoplasmosis with 2 positive result (6.67%) from mother's blood (samples 5 and 21) and 3 positive result (10%) from fetal tissue (samples 5, 20 and 21). The lower incidence of toxoplasmosis was obtained in women at age below 20 with 4 positive results, each 2 positive results (6.67%) from mother's blood and fetal tissue (samples 26 and 30). In women aged 30-39, there were also 4 positive results, 1 positive result (3.33%) from mother's blood (sample 24) and 3 positive results (10%) from fetal tissue (sample 11, 15 and 24). In older women over 40, only 1 positive result (3.33%) was found from fetal tissue (sample 12). According to this, we can see that the highest *Toxoplasma gondii* infection occurs especially in active reproductive women, aged between 16 to 39 and the incidence of infection decreases with age increase. It gives a perception that there is a lower toxoplasmosis incidence in older patients (in this study older than 40) because older patients have a bigger chance to get a contact with *Toxoplasma gondii* which gives a higher anti-body (IgG) protection to recurrent infection. This assumption still need further study with larger subject sample with ELISA assay as a quantitative screening tool. ELISA can give more accurate result than agglutination test be-cause it can describe IgG antibody level, which gives protection to recurrent infection.[25,26]

According to gestational age, the largest positive result was found in gestational age $\geq$ 8-12 weeks, with 4 positive cases (13.33%) from mother's blood and 8 positive cases (26.67%) from fetal tissue. Four positive cases from

mother's blood consisted of sample 21 and 30 (8-10 weeks) and 24 and 26 (10-12 weeks), while 8 positive cases from fetal tissue consisted of sample 11, 12, 24 and 26 (10-12 weeks) and sample 15, 20, 21 and 30 (8-10 weeks). We only found 1 positive case (3.33%) in gestational age < 8 weeks (sample 5), both on mother's blood and fetal tissue and no positive result at gestational age over 12 weeks. This result shows that the highest spontaneous abortion occurred at 8-12 weeks gestational age, which becomes the most criti-cal period. It support the suggest-ion made in some previous studies, which used serological assay, that toxoplasmosis has a significant correlation with spon-taneous abortion in the first trimes-ter of pregnancy and the rate of spontaneous abortion will increase if toxoplasmosis occurs at early gestational age.[27,28,29] The correlation is so strong because the overall positive result for mother's blood were 5 cases (16.66%) and 9 cases for fetal tissue (30%). Further study with larger sample is needed to support the "evidence based medicine" principle.

Beside age and gestational age, spontaneous abortion history and parity were some parameters we used. In spontaneous abortion history, the highest number of positive cases was on women with first spontaneous abortion history, consisting of 5 positive cases (16.67%) from mother's blood (sample 5, 21, 24, 26 and 40) and 7 positive cases (23.33%) from fetal tissue (sample 5, 11, 20, 21, 24, 26 and 40). We found only 1 positive case from fetal tissue (sample 15) from mother with more than 3 times (5 times) spontaneous abortion history. Some previous studies using serology method suggested that it still need some further studies with larger sample in toxoplasmosis patients with recurrent abortion.[12,25]

In this study, there are 5 positive cases on mother's blood (16,67%) and 9 positive cases on fetal tissue (30%). In fact, those 5 positive cases from mother's blood also give positive result in their fetal tissue samples and the remaining 4 positive cases only occurred on fetal tissue. There are some possibilities that could cause this result as following:

1. Late phase of acute infection (early phase of chronic infection)

   In this phase, the antibody already increased, reached the peak state and started to going down. The higher level of antibody will give a positive result in serology test. The positive result in mother's blood shows that the parasite still is present in circulation, while in trophozoite or bradyzoite form. Trophozoite is the acute form of *Toxoplasma gondii* while bradyzoite is the chronic from of the parasite and evolve after the parasite penetrates the white blood cell and forms a protective specific vacuole. The

positive result on fetal tissue shows that the parasite already penetrated fetal tissue, in this case placenta, and is followed by cyste formation causing abortion to occur.[30,31,32]

2. Toxoplasmosis reinfection
   Some literature mentioned that *Toxoplasma gondii* infection gives a total protection to a new infection. Based on the re-sult above, especially on 5 posi-tive cases on mother's blood and fetal tissue, there is a possibility that the protection does work but not totally. This gives a chance for a new infect-ion to occur. The previous To-xoplasma gondii infection was shown by the cyst form in placenta that gives a positive result on fetal tissue while the new infection was shown by the presence of the parasite in blood circulation giving a positive result on mother's blood.[3,12,23,24,27]
3. Toxoplasmosis infection re-lapse
   The parasite cyst in tissue can rupture and release highly in-vasive trophozoite into blood circulation. This situation deve-lops in patients with decreas-ing immune reaction. Cyst already formed in tissue is shown by positive result in fe-tal tissue and newly develop-ing trophozoite in circulation is shown by positive result in mother's blood.[2,3,35]

The remaining 4 positive cases occuring only in fetal tissue showed that the parasite only presented in cyst form and there are no trophozoite and bradyzoite in systemic circulation.

Nested PCR method used in this study eliminated false positive possibility because it has a better sensitivity and specificity than an ordinary PCR method. This study has no comparable stu-dies because there were no pre-vious published studies that used nested PCR to detect *Toxoplasma gondii* in blood and tissue. This study found a quite higher positive result, 16.7% positive result was found in mother's blood and 30% in fetal tissue. There is only one other similar study titled "Identifi-cation of *Toxoplasma gondii* B1 gene with PCR in aborted fetus preserv-ed in formalin", conducted by M. Assmar et al from Teheran, Iran, published in *Irn J Med Sci 2000; 25 (1&2): 59-61*. This study found a 20% positive result in fetal tissue.[23]

Summary

Based on the result, we no-ticed that the incidence of *Toxo-plasma gondii* infection in women with spontaneous abortion is very high at RSUP Manado. From 30 patients, there are 5 positive cases from mother's blood (16,67%) and 9 positive cases (30%) from fetal tissue that suggested a strong correlation between abortion in early pregnancy with toxoplas-mosis, evidence by a very high positive PCR result in fetal tissue at early gestational rate.

Furthermore, this study also showed PCR capability to detect *Toxoplasma gondii* both in mother's blood and fetal tissue. It proved that PCR has a very high sensitivity and specificity because it can detect the parasite in white blood cells that constitute only a small part of the total blood. It also showed that the PCR is enabling the clinician to estimate infection period, whether it is in acute or chronic period.

In this study, serological assay and PCR were done at the same time so the exact decision whether the patient had been infected or is still in the infection period could not be made. Another problem is the serological test used to detect IgG antibody which was not a quantitative method. Based on those problems, further study with more subjects and larger sample and more sensitive quantitative method like ELISA combined with PCR is needed. [12,36-38]

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APPENDIX

TABLE 1. Primers used for first and second round PCR.

Primer	Sequence
First primer	5'-CCATGCATGTCTAAGTATAAGC-3' 5'-GTTACCCGTCACTGCCAC-3'
Second primer	5'-CTAAGTATAAGCTTTTATACGGC-3' 5'-TGCCACGGTAGTCCAATAC-3'

TABLE 2. PCR result for women with spontaneous abortion characterized by age.

No	Age (y)	Latex Agglutination		PCR			
				Mother's blood		Fetal tissue	
		(+)	%	(+)	%	(+)	%
1	< 20	4	13.33	2	6.67	2	6.67
2	20 – 29	13	43.33	2	6.67	3	10.00
3	30 – 39	8	26.67	1	3.33	3	10.00
4	> 40	5	16.67	0	0.00	1	3.33
	Total	30	100.00	5	16.67	9	30.00

TABLE 3. PCR result for women characterized by gestational age.

No	Gestational age (weeks)	Latex Agglutination		PCR			
				Mother's blood		Fetal tissue	
		(+)	%	(+)	%	(+)	%
1	< 8	2	6.67	1	3.33	1	3.33
2	≥ 8 – 12	21	70.00	4	13.33	8	26.67
3	≥ 12 – 16	7	23.33	0	0.00	0	0.00
4	≥ 16 – 20	0	0.00	0	0.00	0	0.00
	Total	30	100.00	5	16.67	9	30.00

TABLE 4. PCR result for women characterized by spontaneous abortion history.

No	Spontaneous abortion history	Latex Agglutination		PCR			
				Mother's blood		Fetal tissue	
		(+)	%	(+)	%	(+)	%
1	0 – 1	27	90.00	5	16.67	7	23.33
2	2 – 3	2	6.67	0	0.00	1	3.33
3	> 3	1	3.33	0	0.00	1	3.33
	Total	30	100.00	5	16.67	9	30.00

TABLE 5. Data analysis with Fischer's Exact Test.

			PCR of Mother's blood		Total
			Negative	Positive	
PCR of Fetal tissue	Negative	Count	21		21
		% PCR JA	100,0%		100,0%
		% PCR DI	84,0%		84,0%
		% Total	70,0%		70,0%
	Positive	Count	4	5	9
		% PCR JA	44,4%	55,6%	100,0%
		% PCR DI	16,0%	100,0%	30,0%
		% Total	13,3%	16,7%	30,0%
Total		Count	25	5	30
		% PCR JA	83,3%	16,7%	100,0%
		% PCR DI	100,0%	100,0%	100,0%
		% Total	83,3%	16,7%	100,0%

TABLE 6. PCR and Serological Test Result from All Samples

Patient no.	Age (y)	Gestation age (week)	Parity	Serologic Test	PCR	
				MS	FT	MB
1	29	8-10	P1A1	+	-	-
2	20	10-12	P0A1	+	-	-
3	25	14-16	P1A1	+	-	-
4	32	10-12	P1A1	+	-	-
5	27	7-8	P1A1	+	+	+
6	35	10-12	P2A1	+	-	-
7	20	10-12	P1A1	+	-	-
8	40	12-14	P3A1	+	-	-
9	18	8-10	P0A1	+	-	-
10	46	12-14	P2A2	+	-	-
11	35	12-14	P2A1	+	+	-
12	41	10-12	P4A5	+	+	-
13	31	8-10	P1A1	+	-	-
14	23	8-10	P1A1	+	-	-
15	32	8-10	PIA2	+	+	-
16	28	8-10	P1A1	+	-	-
17	29	8-10	P0A1	±	-	-
18	24	8-10	P1A1	+	-	-
19	21	8-10	P1A1	+	-	-
20	23	8-10	P0A1	+	+	-
21	25	8-10	P1A1	+	+	+
22	16	10-12	P0A1	+	-	-
23	39	6-8	P4A1	±	-	-
24	35	10-12	P4A1	+	+	+
25	40	12-14	P3A1	+	-	-
26	19	10-12	P0A1	+	+	+
27	30	10-12	P1A1	+	-	-
28	40	14-16	P2A1	+	-	-
29	20	12-14	P0A1	+	-	-
30	16	8-10	P0A1	+	+	+

FT : Fetal tissue
MB : Mother's blood
MS : Mother' serum

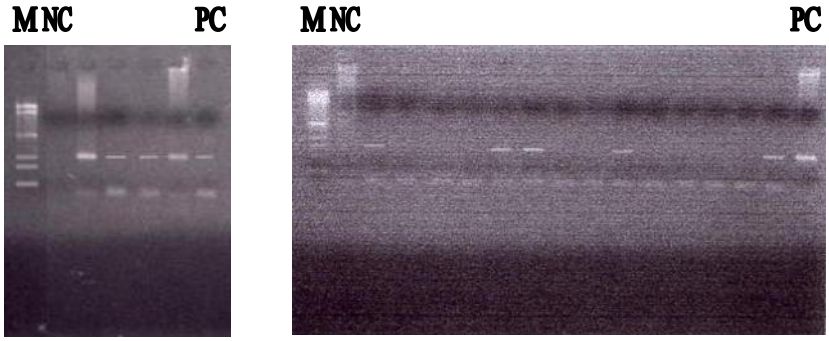

Figure 1 and 2. Positive result of nested PCR from fetal tissue.
Figure 1 (left) showed 4 positive result from sample 11, 12, 15 and 20 whenever figure 2 (right) showed 5 positive result from sample 5, 21, 24, 26 and 30.

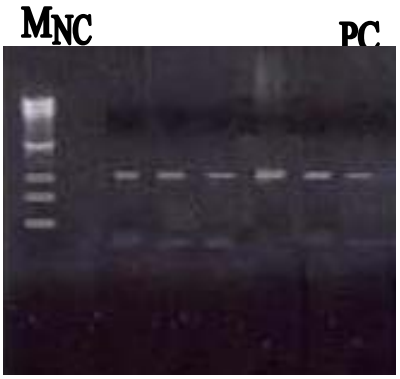

Figure 3. Positive result of nested PCR from mother's blood.
Figure 3 showed 5 positive result from sample 5, 21, 24, 26 and 30.