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A cohort study of maternal screening for congenital *Toxoplasma gondii* infection: 12 years' experience

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Running title: Toxoplasma screening in pregnancy

Abstract

Primary infection with *Toxoplasma gondii* (*T. gondii*) during pregnancy may cause congenital infection of the infant. This study evaluated whether screening using IgG avidity and multiplex-nested polymerase chain reaction (PCR) methods was effective for detecting a high-risk pregnancy for congenital *T. gondii* infection. In a prospective cohort study serum *T. gondii* IgG avidity was measured in 469 pregnant women who had a positive test for *T. gondii* antibody plus a positive or equivocal test for IgM. Multiplex-nested PCR for *T. gondii* DNA on amniotic fluid, maternal blood, and neonatal blood was performed with informed consent. Low (<30%), borderline (30-35%), and high (>35%) IgG avidity indices were found in 104 (22.2%), 30 (6.4%), and 305 (71.4%), respectively. A total of 12 cases had a positive PCR test for amniotic fluids of the prenatal amniocentesis or at birth, or neonatal blood. Seven of the 12 cases were diagnosed as having congenital *T. gondii* infection, and they had low IgG avidity indices. Congenital *T. gondii* infection screening using of IgG avidity and multiplex-nested PCR methods for pregnant women with a positive test for *T. gondii* antibody plus a positive or equivocal test for *T. gondii* IgM was useful for detecting a high-risk pregnancy and diagnosing congenital *T. gondii* infection.

Key words:

Introduction

Toxoplasma gondii (*T. gondii*) infection is one of the most common protozoan parasitic infections in <u>human</u> and other warm-blooded animals. It is caused by ingesting of raw and undercooked meat, which carries tissue cysts, by consuming infected water and food, or by accidental intake of contaminated soil. Vertical transmission from mothers with primary infection during pregnancy to their fetuses may lead to congenital *T. gondii* infection that causes choroidoretinitis, intracranial calcification, hydrocephalus, and mental retardation of the infant.

Serologic tests of IgM antibody are commonly performed to diagnose acute *T. gondii* infection. However, a false-positive result frequently occurs [1], and *T. gondii* IgM antibody may persist for many months or even years following the acute infection [2, 3]. Therefore, a confirmatory test of IgG avidity is often necessary [1, 4]. A low IgG avidity index assists in diagnosing primary *T. gondii* infection during pregnancy and identifying a high-risk pregnancy for congenital *T. gondii* infection [5].

Congenital *T. gondii* infection is diagnosed based on the presence of *T. gondii* DNA in the amniotic fluid and neonatal blood by polymerase chain reaction (PCR) methods. B1 gene has been used as a standard for *T. gondii* PCR analysis [6-8]. We have developed a multiplex-nested PCR method enabling semi-quantitation of *T. gondii* gene copy numbers [9]. A prospective cohort study of *T. gondii* infection screening, wherein *T. gondii* IgG

avidity was measured in pregnant women with a positive or borderline test for *T. gondii* IgM, was conducted. We have reported the preliminary results of the efficacy of maternal screening for congenital *T. gondii* infection [9]. The present study extended the cohort study and reported 12 years' experience of congenital *T. gondii* infection screening using IgG avidity and multiplex-nested PCR methods

Materials and Methods

Patients

This prospective cohort study was approved by the institutional review board of Kobe University Hospital. Informed consent was obtained from all of the participants. Between April 2005 and July 2017, pregnant women with a positive test for *T. gondii* antibody [hemagglutination (HA) or IgG], together with a positive or equivocal for *T. gondii* IgM were included in this study. To further assess acute *T. gondii* infection, 469 pregnant women who tested positive for *T. gondii* antibody in the peripheral blood obtained at 9-14 weeks of gestation (GW) and also tested positive or equivocal for *T. gondii* IgM a few weeks later were referred to NTT East Sapporo Medical Center or Kobe University Hospital. *T. gondii* antibodies were measured by using of *T. gondii* HA antibody (TOXO-HA, Japan lyophilization laboratory, Tokyo, Japan) or *T. gondii* IgG antibody (PLATERIA Toxo IgG, Bio-Rad, Tokyo, Japan; Access TOXO IgG, Beckman Coulter,

Tokyo, Japan), and *T. gondii* IgM (PLATERIA Toxo IgM, Bio-Rad, Tokyo, Japan). All

women underwent serum *T. gondii* IgG avidity measurements based on congenital *T. gondii*

infection *s*creening.

Congenital T. gondii infection screening

Figure 1 shows the scheme of congenital *T. gondii* infection screening in this study.

The 469 pregnant women with a positive test for *T. gondii* antibody plus a positive or

equivocal test for *T. gondii* IgM underwent serum *T. gondii* IgG avidity measurements.

When IgG avidity index was less than 30%, primary infection during pregnancy or

periconception period was strongly suspected; and women received cyclic administration of

acetylspiramycin (1.2 g/day for three weeks, followed by an interval of no medication for

two weeks) until delivery [9]. Women with 30-35% IgG avidity index of a borderline were

recommended to receive the acetylspiramycin therapy. When IgG avidity index was more

than 35%, non-primary infection was suspected, women did not receive the therapy unless

the women desired the medication.

Multiplex-nested PCR for *T. gondii* DNA in the peripheral blood was performed in

addition to IgG avidity measurement when women agreed. When the blood was tested

positive for multiplex-nested PCR, the women received the acetylspiramycin therapy

regardless of IgG avidity results. Amniocentesis followed by multiplex-nested PCR was

performed with informed consent when the women desired it because of suspected primary infection or anxiety. If the amniotic fluid tested positive for multiplex-nested PCR, in addition to the acetylspiramycin therapy, the women received administration of pyrimethamine (25-50mg/day) and sulfadiazine (500-1000mg/day) until 27 GW.

At birth, *T. gondii* IgM in neonatal blood was measured in all subjects except cases of induced abortion, and multiplex-nested PCR for neonatal blood and/or amniotic fluid was performed when informed consent was obtained. The presence of congenital infection was assessed for all infants by ophthalmofundoscopy, cerebral ultrasound, physical and neurological examinations. A head CT scan was used if necessary.

Congenital infection was diagnosed by any of the following: 1) Increase in anti- *T. gondii* IgG titer during the first year of life or increasing IgG titer compared with the mother's; 2) positive IgG with positive IgM; 3) positive *T. gondii* PCR test in cerebrospinal fluid, blood or urine; 4) positive IgG beyond 12 months of age, and 5) infants with characteristic clinical findings, positive IgG, but negative IgM [10].

IgG avidity measurement

Serum *T. gondii* IgG avidity was measured by using *T. gondii* antigen-coated wells, Enzygnost® Toxoplasmosis/IgG (DADE Behring, Marburg, Germany). Briefly, diluted serum with a sample buffer was added to the antigen-coated wells in duplicate. After the

antigen-antibody reaction, one of the wells was treated with PBS containing 0.1% BSA, 0.05% Tween 20 (reaction buffer) and the other well was treated with reaction buffer containing 8 M urea. After 30 minutes incubation at room temperature, anti-*T. gondii* IgG was detected by horseradish peroxidase-conjugated anti-human IgG. Thereafter, these wells were stained with substrate reagent (tetramethylbenzidine plus Substrate-Chromogen; DAKO, Carpinteria, CA), and absorbance at 450 nm (OD_{450}) was measured. The avidity index (AI) was calculated using the following formula.

AI (%) = 8 M urea-treated OD_{450} values/ untreated OD_{450} values x 100

The coefficients of variation were below 10% in intra-assay and below 15% in inter-assay.

Multiplex-nested PCR method

To assess the presence of *T. gondii* DNA, multiplex-nested PCR for maternal blood, neonatal blood and amniotic fluid was performed with informed consent. The primer pairs were designed for different four genes of *T. gondii* including B1 (GenBank accession number, AF179871), cyclin dependent kinase (cdk, AJ534295), SAG5E (AY363043), bradyzoite surface antigen (BSR4, AF394603). These four genes were simultaneously amplified in one tube reaction by the multiplex PCR using Multiplex PCR Kit (QIAGEN GmbH, Hilden, Germany). As a nested PCR method, the primer pairs in the second multiplex PCR were designed so that each of the PCR products should be 5 bp smaller than

that of the first multiplex PCR. These primer sequences are shown elsewhere [11]. The four recombinant genes were used as positive controls (Hokkaido System Sciences, Sapporo, Japan). When there were more than approximately 100 gene copies, these four bands were detected after the first PCR reaction. After the second PCR reaction, the four bands were detected if there were more than approximately 10 gene copies of *T. gondii*. Thus, *T. gondii* gene copy numbers in the samples were estimated semi-quantitatively [9].

Results

Of the 469 pregnant women with a positive test for *T. gondii* HA/IgG plus a positive or equivocal test for *T. gondii* IgM, 104 (22.2%) had low IgG avidity (<30%), 30 (6.4 %) had borderline avidity (30-35%), and 335 (71.4%) had high avidity (>35%) results.

Of 295 pregnant women who underwent multiplex-nested PCR for *T. gondii* DNA in the peripheral blood, six (2.0%) had positive results. Of 47 women who underwent prenatal amniocentesis followed by PCR test at 16-30 GW, five (10.6%) had positive results. At birth, the amniotic fluids for PCR test were obtained from 172 women, and nine (5.2%) had positive results. Of 224 pregnant women who underwent multiplex-nested PCR for *T. gondii* DNA in neonatal blood, four (1.8%) had positive tests. A total of 12 cases had a positive PCR test for amniotic fluids of the prenatal amniocentesis or at birth, or neonatal blood. All the 12 women had low IgG avidity of 2-29%. Five of the 12 cases had a positive

PCR test for amniotic fluids of the prenatal amniocentesis or at birth, but not for neonatal blood. These five cases tested negative for *T. gondii* IgG and IgM at 12 months old and were diagnosed with no congenital infection.

Table 1 shows a total of seven pregnancies with diagnoses of congenital *T. gondii* infection of the fetuses. All the seven women presented low IgG avidity results, and six women prenatally received the acetylspiramycin therapy. Case 1 had positive PCR tests for both amniotic fluids of the prenatal amniocentesis and at the birth. She received the acetylspiramycin therapy from 22 GW until delivery, and the pyrimethamine and sulfadiazine therapy between 25 and 28 GW. The infant with intracranial calcifications detected by a CT scan received a therapy with pyrimethamine and sulfadiazine for one year. The infant tested positive for *T. gondii* IgG and IgM at 18 months old [11]. Currently, the 12-year-old boy has no physical or neurological abnormality. Case 2 had a positive PCR test for amniotic fluid of the prenatal amniocentesis. This pregnancy ended in the induced abortion at 21 GW. The couple did not desire an autopsy or the further examination. Case 3 had a positive PCR test for amniotic fluid at the birth but not of the prenatal amniocentesis. This infant tested positive for *T. gondii* IgG at 12 months old and IgM at four months old. Currently, the nine-year-old boy has no physical or neurological abnormality without medication.

Case 4 had positive PCR tests for amniotic fluid at the birth and neonatal blood. Case 5 also had positive PCR tests for amniotic fluid at the birth and neonatal blood. Case 6 had negative PCR tests for both amniotic fluids of the prenatal amniocentesis and at the birth, but had a positive PCR test for neonatal blood. Case 7 had a positive PCR test for neonatal blood. All four infants form Cases 4-7 tested negative for *T. gondii* IgG and IgM at 12 months old, and had normal development thereafter.

Of six women with positive PCR results in the peripheral blood, only one woman had a low IgG avidity index and congenital *T. gondii* infection of the neonate (Case 1). *T. gondii* IgM in neonatal blood was usually measured at birth, however, none had a positive result in the present study. None of 30 women with borderline IgG avidity and 335 women with high avidity results had a positive PCR test for amniotic fluids of the prenatal amniocentesis or at birth, or neonatal blood. None of neonates from these mothers had congenital *T. gondii* infection.

Discussion

The present cohort study of the 12 years' experience of maternal *T. gondii* antibody screening demonstrated an incidence of congenital *T. gondii* infection as 1.5% (7/469) in Japanese women with a positive test for *T. gondii* HA/IgG plus a positive or equivocal test for *T. gondii* IgM. One hundred four (22.2%) women who presented low IgG

avidity were supposed <u>to have</u> primary infection of *T. gondii* during the first trimester. An incidence of congenital *T. gondii* infection in Japanese women who acquired primary infection during pregnancy was calculated as 6.7% (7/104). None of women with a high or borderline IgG avidity index had a positive PCR test for *T. gondii* DNA in the amniotic fluid or neonatal blood. Conversely, all seven women with diagnoses of congenital *T. gondii* infection had low IgG avidity indices. It was confirmed that IgG avidity measurements for women with a positive test for *T. gondii* HA/IgG plus a positive or equivocal test for *T. gondii* IgM were useful to detect a high-risk pregnancy for congenital *T. gondii* infection <u>with high sensitivity and high specificity</u>.

The prevalence of *T. gondii* antibody was reported as 16.4% in Japanese adults [12], and 10.3% in pregnant women from Miyazaki prefecture [13]. A Japanese literature reported the prevalence of *T. gondii* antibody as 3.63% and *T. gondii* antibody plus *T. gondii* IgM as 0.60% in 1,848 pregnant women from Hokkaido prefecture [14]. In the present study, 22.2% of pregnant women with a positive test for *T. gondii* antibody plus a positive or equivocal test for *T. gondii* IgM were supposed to acquire primary infection of *T. gondii* due to their low IgG avidity index. Therefore, it was estimated that at least 0.13% of Japanese pregnant women acquired primary infection of *T. gondii* during the first trimester. We did not evaluate seroconversion of *T. gondii* antibody during the second and third trimesters. Therefore, the actual frequency of primary *T. gondii* infection during pregnancy

may be higher than the above estimated percentage. The incidence of congenital *T. gondii* infection in Japan was estimated to be 0.009% in Hokkaido and 0.026% in Miyazaki prefectures (0.9-2.6/10,000 births in Japan) under the condition that all pregnant women underwent congenital *T. gondii* infection screening and received medications if necessary. The estimated incidence of congenital toxoplasmosis in Japan was consistent with the incidences (1-10 /10,000 live births) reported in the US [15] and Europe [16].

The present cohort study identified seven cases with congenital *T. gondii* infection based on diagnostic criteria which were authorized by the study of the Japan Agency for Medical Research and Development (No. 17gk0110021h0002) in 2018 and used for clinical practitioners of Japan Society of Obstetrics and Gynecology and Japan Pediatric Society. Congenital *T. gondii* infections of Cases 4-7 were diagnosed due to a single finding of a positive test for *T. gondii* PCR in neonatal blood. However, the four infants did not present seroconversion of *T. gondii* IgG or IgM at 12 months old. Neither clinical findings of congenital infection nor serological confirmation of *T. gondii* infection was demonstrated. The gene copy number was very small, at most, several copies in the neonatal blood of four infants. There might be a false positive result of a PCR test for congenital *T. gondii* infection. Therefore, infants must be followed up at least one year of age to confirm congenital infection serologically.

The present study has some limitations. The definite infection was not confirmed in Case 2, because the couple did not consent to any further examination after the induced abortion. The quality of the PCR method, false positive and false negative rates are still unclear. In conclusion, congenital *T. gondii* infection screening with a combination of IgG avidity in the maternal blood and multiplex-nested PCR in the amniotic fluid and neonatal blood was useful for detecting a high-risk pregnancy and diagnosing *T. gondii* infection. The findings of the present study will provide new perspectives on the clinical management of congenital *T. gondii* infection.

Potential conflicts of interest

All authors report no potential conflicts of interest.

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Legend

Figure 1.   Maternal screening for congenital *Toxoplasma gondii* infection

Figure 1
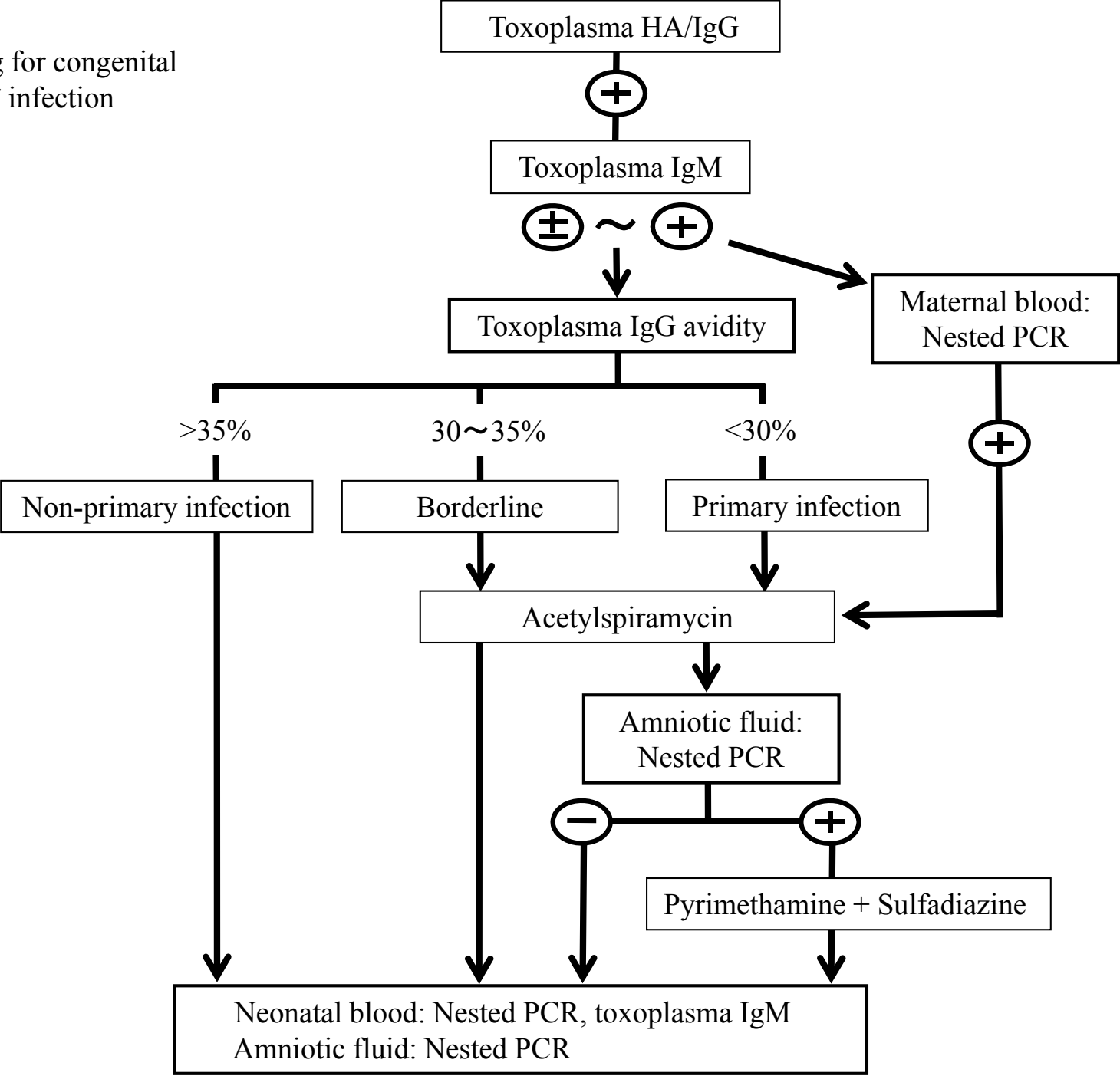Maternal screening for congenital
Toxoplasma gondii infection

Table 1 Seven pregnancies with diagnoses of congenital *Toxoplasma gondii* infection

Case number	Maternal toxoplasma IgM index (gestational week)	Maternal toxoplasma IgG avidity % (gestational week)	Maternal therapy (gestational week)	Amniotic fluid-PCR at amniocentesis (gestational week)/ estimated gene copy number	Amniotic fluid-PCR at birth (gestational week)/ estimated gene copy number	Birth weight (g) (gestational week)/ sex	Neonatal blood-PCR/ estimated gene copy number	Toxoplasma IgG/IgM (month-old)	Outcome
1	2.8 (17)	23 (28), 71 (8 months postpartum)	Acetylspiramycin (22-38), Pyrimethamine and Sulfadoxine (25-28)	Positive (28)/10-100	Positive/ 10-100	2916 (38)/male	Negative	Positive (18)/Positive (18)	12 years old; congenital toxoplasmosis, 1 year-medication, no sequela
2	0.9 (13)	20 (17)	Acetylspiramycin (14-17)	Positive (17)/10-100	ND	600 (21)/ female	ND	ND	Termination of pregnancy
3	4.6 (15)	4 (15), 20 (21)	Acetylspiramycin (15-38)	Negative (18)	Positive/ 10-100	3220 (38)/male	Negative	Positive (12)/Positive (4)	9 years old; no medication, no sequela
4	1.3 (20)	7 (20)	Acetylspiramycin (19-37)	ND	Positive/ ≥100	2984 (37)/ female	Positive/ 1-several	Negative (12)	7 years old; normal development
5	6.7 (16)	2 (18), 7 (23)	Acetylspiramycin (16-40)	ND	Positive/ several	2810 (40)/ male	Positive/ several	Negative (12)	3 years old; normal development
6	6.8 (9)	9 (12), 7 (25)	Acetylspiramycin (11-38)	Negative (16)	Negative	3150 (38)/ male	Positive/ 1-several	Negative (12)	2 years old; normal development
7	>3.0 (37)	29 (2 days postpartum)	None	ND	ND	3676 (40)/ male	Positive/ several	Negative (12)	2 years old; normal development

ND, not determined.