Parasitology International 64 (2015) 603–608

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Congenital toxoplasmosis and pregnancy malaria detection post-partum: Effective diagnosis and its implication for efficient management of congenital infection

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ARTICLE INFO

Article history:
Received 21 April 2015
Received in revised form 25 July 2015
Accepted 7 August 2015
Available online 8 August 2015

Keywords:
Toxoplasma gondii
Plasmodium falciparum
Placenta
Congenital toxoplasmosis
Pregnancy malaria

ABSTRACT

Congenital toxoplasmosis (CT) and pregnancy malaria (PM) have been individually reported to cause severe negative outcomes in pregnancies but the diagnostic method is still debatable. This study sought to estimate the prevalence of PM and CT single and co-infections in pregnant women by using various specimens including plasma and placental tissues. Genomic DNA extracted from the placenta, cord blood or blood of mothers was tested by PCR. Conventional method of immunodiagnosis was done for CT. We tested 79 pregnant women aged 18–42 years (mean: 28 ± 1.06). Prevalence of *Plasmodium falciparum* infection determined by PCR on mother's peripheral blood specimen was 6.3% whiles 57.3% was recorded for placental tissues ($p < 0.01$). PCR testing for placental tissues showed 29.2% positive for *Toxoplasma gondii*, whiles 76.0% of mothers had serum IgG against *T. gondii*. It should be noted that 6.3% of the placental tissues showed PCR positive for SAG 3, a marker of active infection in *T. gondii*. Although there were no enhanced foetal disorders at birth in our study, there is a possibility of active transmission of *T. gondii* from mothers to foetuses even in immune mothers. Our study suggests that foetuses were exposed to *P. falciparum* and *T. gondii* in utero, and placenta is a better specimen for PCR in detecting such episodes. In cases of PCR-positive samples, clinical follow-up after birth may be important.

1. Introduction

Malaria and toxoplasmosis are parasitic diseases that have individually been implicated in causing severe negative consequences in pregnancy [1–3]. During pregnancy, complex physiological conditions occur thus enabling the mother to fully adapt to the environment. Immune-suppression is noted to be a physiological change that the body of the mother induces for the prevention of foetal rejection as the foetus is considered as an allograft. This, therefore, makes the pregnant woman susceptible to many opportunistic infections [4]. Systemic and opportunistic parasites such as *Plasmodium falciparum* and *Toxoplasma gondii* acquired during pregnancy have the ability to permeate the placenta and serve as the main parameter to establish transmission to the foetus [2,5,6].

Pregnancy malaria (PM) due to *P. falciparum* is noted as a major cause of morbidity and mortality in women in Sub-Saharan Africa but is difficult to diagnose [7–9] due to parasite sequestration and attachment to receptors in the placenta bed [10,11]. In Ghana, standard diagnosis of PM is by microscopic examination of peripheral blood smears (BS) and rapid diagnostic tests (RDTs). These diagnostic protocols are however fraught with challenges, in that most pregnant women remain asymptomatic especially in malaria endemic regions [12] and BS and RDTs often appear negative, leading to chronic untreated PM with insidious consequences that include but not limited to low birth weight (LBW), severe anaemia and hypertension.

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a wide range of hosts, including humans. Infections in humans are usually asymptomatic [1]. Primary infection of *T. gondii* during pregnancy can result in the vertical transmission of tachyzoites which can have severe consequences on the foetus such as retinochoroiditis, hydrocephalus and intracranial calcification [13]. Standard diagnostic protocol for toxoplasmosis is the detection of anti-*T. gondii* antibodies in the sera of patients [14,15]. However, the parasitological examination of the placenta at birth by polymerase chain reaction (PCR) as a tool for diagnosing neonatal congenital toxoplasmosis is known to be one of the most effective methods [16]. In Ghana, there is no approved protocol

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http://dx.doi.org/10.1016/j.parint.2015.08.004
1383-5769/

for the routine diagnosis and management of toxoplasmosis in pregnancy.

The accuracy of diagnostic methods to detect *P. falciparum* and *T. gondii* infections during pregnancy is critical for the management of these diseases [17,18]. Diagnostic performance assessed in non-pregnant populations cannot necessarily be extrapolated to the detection of CT and PM because of factors such as parasite biomass concentrations in tissues [19] and haemodilution [20]. Reports of increased resistance of sulphadoxine–pyrimethamine (SP) across Africa [21] thus increase the need to have more accurate diagnostic approach.

In this study we examine the prevalence of PM and CT single and co-infections in pregnant women by detection of *P. falciparum* from placental tissues and peripheral blood specimen using PCR and; detection of anti-*T. gondii* infection antibodies in plasma and parasite DNA from placental tissues by PCR. We then discuss the better, if not the best, diagnostic method for CT and PM.

2. Materials and methods

2.1. Ethics and sampling permits

The study was reviewed and approved by the Scientific and Technical Committee of Noguchi Memorial Institute for Medical Research and ethical approval was given by the Institutional Review Board of NMIMR (NMIMR-IRB 093/11-12). Written informed consent was obtained from pregnant women who were recruited into the study prior to delivery. Emphasis was placed on the fact that participation was entirely voluntary and there was the liberty of withdrawing from the study at any time without further obligation.

2.2. Study site

The Labour Ward, Department of Obstetrics and Gynaecology at the Korle Bu Teaching Hospital (KBTH) in Accra, Ghana, served as the recruitment site for study participants.

2.3. Study population and participants

Study population comprised of volunteer pregnant women of child bearing age in the third trimester of pregnancy attending Ante-Natal Care at the Obstetrics clinic of KBTH who were enrolled to participate and, when they went into labour were involved in the study. Women who volunteered whilst on admission to the Labour Ward were also included.

2.4. Blood and placenta tissue sample collection

From the cubital veins of pregnant women 2 ml of venous blood was drawn immediately after expulsion of placenta using a sterile hypodermic syringe and placed into labelled 6 ml vacuette® tubes containing citrate. In cases where intravenous blood could not be drawn from the mothers, due to conditions such as excessive loss of blood and placing into intensive care, an incision was made in the inter-villous space of the utero-placental region (maternal side) and up to 5 ml of blood was drawn from placenta basal plate endometrial arteries. Up to 5 ml of cord blood was also drawn from the umbilical vein of the feto-placental region (foetus side) of the placenta into 6 ml vacuette® tubes containing citrate to represent the neonates. Plasma was obtained from each blood sample by centrifugation at 14,000 rpm for 10 min and stored at −40 °C. Blood blots were also prepared on Whatman No. 3 filter papers (GE Healthcare Life Sciences, Japan) from blood of the mothers. About 0.5 ml of blood drops were blotted on the labelled filter paper and allowed to air-dry. These were then placed into labelled zip-lock bags, transported to the laboratory and stored at 4 °C until use.

A small incision was made 5 cm beneath the utero-placental region (mother side) with a sterile surgical scissors; about 5 g of placental tissue was cut and placed in a labelled tube containing phosphate buffered saline (PBS). Care was taken to ensure that there was no cross contamination of the samples collected. These were then transported to the laboratory and stored in a freezer at −20 °C until use.

2.5. Genomic DNA extraction from peripheral blood and placenta tissues

Genomic DNA was extracted from blood (maternal and foetal) and placental tissue samples using DNeasy® blood and tissue kit (QIAgen, USA) following manufacturer's instructions.

2.6. Detection of P. falciparum by PCR

Previously extracted and purified DNA was amplified using a nested PCR method adopted and modified from a previously published protocol [22].

Generic *Plasmodium* forward (5′CCTGTTGTTGCCTTAAACTTC3′) and reverse (5′TTAAAATTGTTGCAGTTAAAACG3′) primers for the outer reaction were used to amplify a 205 bp region of *P. falciparum* 18SrRNA gene by PCR. 5 μl of DNA, 1 × PCR buffer, 2.5 μM $MgCl_2$, 0.2 mM dNTP mix with 10 μM each of forward and reverse primers in a total volume of 25 μl was amplified in a PCR thermo-cycler (Takara Shiga, Japan). Known positive samples from previously malaria diagnosed individuals served as a positive control. Reaction conditions were initial denaturation at 94 °C for 2 min, followed by denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min followed by another 5 min at 72 °C for a total of 35 cycles.

2.7. Detection of T. gondii DNA in placental tissues by PCR

A set of unlinked two-way markers SAG 3 and GRA 6 was used for the amplification of specific gene regions of *T. gondii* using a modification of the nested polymerase chain reaction (nPCR) method previously described [23–25].

SAG 3 primers {outer reaction primers; P43S1: CAACTCTCACCATTCC ACCC; P43AS1: GCGCGTTGTTAGACAAGACA and inner reaction primers; P43S2: TCTTGTCGGGTGTTCACTCA; P43AS2: CACAAGGAGACCGAGAAG GA} were used to amplify the 225 bp region of the SAG 3 (p43) single copy gene encoding the *T. gondii* surface antigen located on Chromosome XII.

GRA 6 primers {outer primers GRA6-F1x: ATTTGTGTTTCCGAGCAG GT; GRA6-R1: GCACCTTCGCTTGTGGTT and inner reaction primers GRA6-F1: TTTCCGAGCAGGTGACCT; GRA6-R1x: CGCCGAAGAGTTGACA TAG} were used to amplify the 344 bp region of the GRA 6 gene located on Chromosome X.

For amplification of each marker target DNA sequence, the first reaction was carried out in a 25 μl volume containing 1 × Gotaq® Green Master Mix (Promega, USA), 1.0 μM each of forward and reverse primers, 7 μl of double distilled water and 5 units of DNA polymerase.

The reaction mixture was subjected to a set of conditions 94 °C for 2 min, followed by denaturation at 94 °C for 30 s, annealing at 58 °C for 1 min, and extension at 72 °C for 2 min followed by another 5 min of overextension at 72 °C for a total of 25 cycles for the first nested reaction.

2.8. Detection of anti-T. gondii antibodies by enzyme linked immunosorbent assay

Plasma sample for each of both mother and foetus was tested for the presence of anti-*T. gondii* IgG and IgM using Enzyme Linked Immunosorbent Assay (ELISA) commercial kits (CTK Biotech, Inc., San Diego, CA, USA) strictly in accordance with manufacturer's recommendations. Briefly, 100 μl (for IgG ELISA) and 50 μl (for IgM ELISA) of sample diluent was added to the test wells of the microtitre plates and 10 μl of each test