The Burden of *Toxoplasma Gondii* In Spontaneous Miscarriage and its Association with Rhoptry Protein 5 Gene and *Toxoplasma gondii* specific primers GRA 6 Gene Expression in Iraq Women

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Abstract

Background: One of the major infectious micro-organism that is associated with spontaneous abortion is toxoplasmosis. Under diagnosis of toxoplasmosis and lack of understanding the exact mechanism and pathogenesis of spontaneous abortion associating toxoplasmosis are in our opinion the main problem facing clinicians who are concerned with categorization and management of spontaneous abortion in our pregnant ladies.

Aim of the current study: to diagnose placental tissues parasitic infections using sophisticated nested PCR method in addition to evaluating gene expression of two pathogenetically Toxoplasma genes, namely ROP-5 and GRA-6, as a step toward estimating the exact prevalence of Toxoplasma associated spontaneous abortion in Iraqi community

Patients and methods: The case control study was based on the inclusion of 101 women with spontaneous abortion and 20 pregnant ladies who succeeded to get pregnant serving as control group. The placental tissues were obtained from the pool of women visiting Al-Kut maternity teaching hospital for purpose of delivery or because of abortion. Those tissues were then processed in central laboratory using conventional PCR technique searching for evidence of *toxoplasma gondii* DNA. Obtained tissues were also subjected to real time PCR for detection of Rhoptry protein 5 gene expression and *Toxoplasma gondii* specific primers GRA 6. The lab work was done according to instruction of providing company.

Results: Positive results were limited to women with spontaneous abortion, that is, none of control women had PCR evidence of *Toxoplasma* DNA in their placental tissues, 72 (71.3 %) versus 0 (0.0 %); the difference was highly significant ($P < 0.001$). An estimation of the risk of abortion accompanying toxoplasmosis has been carried out in terms of approximate Odds ratio and the results has been strikingly high (100.8). Both Rhoptry protein 5 and *Toxoplasma gondii* specific primers GRA 6 gene expressions have been significantly correlated to serum IgG and IgM positive tests in a negative way.

Conclusion: PCR carried out on placental tissue is significantly higher sensitive in detecting toxoplasma gondii than serology and Both Rhoptry protein 5 and *Toxoplasma gondii* specific primers GRA 6 gene expressions play significant role in spontaneous abortion.

Key words: *spontaneous abortion, Rhoptry protein 5, Toxoplasma gondii specific primers GRA 6 gene expressions*

Introduction

The problem of spontaneous abortion is relatively common in our community as well as in several regions around the world [(1)]. The problem of spontaneous

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abortion in addition to infertility account for the major obstacles that every newly married couple may face during their planning to build up their families [2]. In addition, spontaneous abortion is associated with a number of obstetric complications that may increase the incidence rate of morbidity and mortality accompanying natural pregnancy [3]. Examples of these complications are retained placental and or fetal parts with risk of bleeding and infection. Indeed, hemorrhage and sepsis are among common associated complications that may threaten the life of every pregnant lady experiencing spontaneous abortion [3]. Therefore, identifying causes and risk factors that may precipitate spontaneous abortion is a crucial step in the prevention and treatment of spontaneous abortion and its associated complications [4].

Infection of the product of abortion is by far one of the principal etiological factors that predispose pregnant ladies to spontaneous abortion. One of the major infectious micro-organism that is associated with spontaneous abortion is toxoplasmosis [5]. Indeed, toxoplasmosis is both common worldwide and in our community [5]. This parasite is responsible for a number of morbidities and mortalities as it can infect a number of human systems and tissue such as nervous system and pregnancy products [6].

Serological investigations are the routine investigations that are carried out in daily obstetric practice in order to diagnose toxoplasmosis in women with recurrent spontaneous abortions [7]. However, the sensitivity and specificity of these serological methods are variable and are far less than that offered by sophisticated molecular investigations [8].

Under diagnosis of toxoplasmosis and lack of understanding the exact mechanism and pathogenesis of spontaneous abortion associating toxoplasmosis are in our opinion the main problem facing clinicians who are concerned with categorization and management of spontaneous abortion in our pregnant ladies [9]. For that reasons, the following study was designed, planned and carried out in order to diagnose placental tissues parasitic infections using sophisticated nested PCR method in addition to evaluating gene expression of two pathogenetically Toxoplasma genes, namely ROP-5 and GRA-6, as a step toward estimating the exact prevalence of Toxoplasma associated spontaneous abortion in Iraqi community and to clarify the role of these two genes in the pathogenesis of spontaneous abortion accompanying toxoplasmosis.

Patients and Method

The case control study was based on the inclusion of 101 women with spontaneous abortion, mostly in the first trimester and some in the second trimester, and 20 pregnant ladies who succeeded to get pregnant serving as control group. The placental tissues were obtained from the pool of women visiting Al-Kut maternity teaching hospital for purpose of delivery or because of abortion. Those tissues were then processed in central laboratory using conventional PCR technique searching for evidence of *toxoplasma gondii* DNA. Obtained tissues were also subjected to real time PCR for detection of Rhoptry protein 5 gene expression and *Toxoplasma gondii* specific primers GRA 6. The lab work was done according to instruction of providing company.

The statistical work was carried out using SPSS version 23 and Microsoft Office Excel 2010. Categorical variables were expressed as number and percentage whereas quantitative variables were expressed as mean and standard deviation. Independent sample t-test was used to compare mean age between study and control groups and chi-square was used to study association between categorical variables. Risk was estimated according to odds ratio and 95 % confidence interval. The level of significance was considered at $P \leq 0.05$.

Results

The statistical analysis in the current study was based on the inclusion of 101 women with spontaneous abortion, mostly in the first trimester and some in the second trimester, and 20 pregnant ladies who succeeded to get pregnant serving as control group. The mean age of women with spontaneous abortion was 29.10 ±7.35 years and that of control group was 25.70 ±5.75 years; despite some difference in mean age, there was no statistical significance ($P = 0.053$). The results of nested PCR results, that have been performed on women retrieved placental tissues, are shown in table 1. Positive results were limited to women with spontaneous abortion, that is, none of control women had PCR evidence of *Toxoplasma* DNA in their placental tissues, 72 (71.3 %) versus 0 (0.0 %); the difference was highly significant ($P < 0.001$), as shown in table 1. Because of the high rate of toxoplasmosis in association with spontaneous abortion (71.3 %), an estimation of the risk of abortion accompanying toxoplasmosis has been carried out in terms of approximate Odds ratio and the results has been

strikingly high (100.8). This implies that any women with placental tissue, toxoplasma invasion is 100 times more liable to develop abortion than women whom placental tissue is free of toxoplasmosis, table 1.

Table 1: Comparison of nested PCR test results between study and control groups

Nested PCR	Abortion group n = 101	Control group n = 20	$\chi 2$	P €	Approximate odds ratio
Positive	72 (71.3 %)	0 (0.0 %)	35.207	< 0.001 HS	100.8
Negative	29 (28.7 %)	20 (100.0 %)			

n: number of cases; €: Fischer exact test; HS: Highly significant at $P \leq 0.05$

Rhoptry protein 5 gene expression in placental tissues obtained from study group is demonstrated in 1. Gene expression up regulation has been observed in 66 (91.5 %) of cases. Gene expression of *Toxoplasma gondii* specific primers GRA 6 in placental tissue of study group has been shown in table 3.13 and figure 3.4. Gene expression down regulation has been observed in 25 (34.7 %); whereas, gene expression up regulation has been recorded in 24 (33.3 %) of cases, as shown in table 3.13. Significant negative association has been observed between the expression of ROP5 and GRA6 as shown in figure 3.4.

Figure 1: Rhoptry protein 5 gene expression and *Toxoplasma gondii* specific primers GRA 6 in placental tissues obtained from study group

Both Rhoptry protein 5 and *Toxoplasma gondii* specific primers GRA 6 gene expressions have been significantly correlated to serum IgG and IgM positive tests in a negative way (negative correlation coefficients, -0.347 and -0.374, respectively and a *P* value of < 0.01), as shown in table 2, implying that the higher the level of serum IgM and IgG, the lower the opportunity for *Toxoplasma gondii* to proliferate and invade placental tissue.

Table 2: Correlations of Rhoptry protein 5 gene expression and *Toxoplasma gondii* specific primers GRA 6 to clinical parameters

Characteristic	ROP5		GRA6	
	r	P	r	P
Age	0.042	0.727	-0.033	0.781
Residency	0.084	0.484	-0.085	0.476
Parity	0.024	0.838	-0.122	0.309
Previous abortions	0.028	0.818	0.127	0.288
Gravidity	0.040	0.740	0.045	0.710
Trimester	0.142	0.236	0.052	0.666
IgG	-0.347	0.003	-0.087	0.465
IgM	-0.374	0.001	-0.215	0.070

Discussion

Although, PCR can be performed on blood as well as serum samples, positive results are usually low, moreover, the presence of positive PCR results using blood or serum is an indirect evidence for a causal relationship between toxoplasmosis and spontaneous abortion [10]; whereas, positive nested PCR results from placental tissues of aborted mothers will be a direct evidence for such causal relationship. For these reasons, the authors of the current study preferred to perform nested PCR on placental tissues over PCR on blood and serum samples.

In the current study, the use of nested PCR significantly increased the detection rate of Toxoplasma parasite in women with spontaneous abortion from 41.6 % to 71.3 % in comparison with serological investigation. In addition, the sensitivity and specificity of both IgG and IgM were low in comparison with nested PCR method. In one study, carried out in Iran on 200 women with spontaneous abortion, the sensitivity of serological test was 53.5 % while that of nested PCR using placental tissue was 10.5 % [8], in disagreement with finding of the current study. Indeed, the low sensitivity and specificity of serologic technique using anti-*toxoplasma* IgG and IgG in comparison with nested PCR using placental tissue has been proved by several other authors [11], [12] and [13] in clear agreement with our findings.

Toxoplasma gondii is an obligate intracellular single-celled parasite that can invade all warm-blooded animals worldwide. During invasion, proteins from parasite organelles such as rhoptry proteins (ROPs) and dense granule proteins (GRAs) are released into host cells and are able to cause significant host damage [14] and [15]. Importantly, maternal *Toxoplasma* infection may give rise to congenital transmission of the parasite to the fetus through the placenta and/or *via* interfering with the immune tolerance on maternal-fetal interface [16], [17] [18] and [19].

Toxoplasma gondii, as many intracellular parasites, is separated from the cytosol of its host cell by a parasitophorous vacuole membrane (PVM). This vacuole forms during host cell invasion and parasite apical organelles named rhoptries discharge proteins that associate with its membrane during this process. ROP5 is not processed during trafficking to rhoptries. We show here that ROP5 is secreted during invasion and associates with the PVM [20]. Based on these facts, the authors of the present study suggested that the association of ROP5 protein with vacuole membrane can be indirectly assessed by studying ROP5 gene expression in placental tissues of women with spontaneous abortion. Molecular investigation in the current study showed upregulation of ROP5 gene expression up regulation has (91.5 %) of cases.

Both Rhoptry protein 5 and *Toxoplasma gondii* specific primers GRA 6 gene expressions have been significantly correlated to serum IgG and IgM positive tests in a negative way (negative correlation coefficients, -0.347 and -0.374, respectively and a P value of < 0.01), implying that the higher the level of serum IgM and IgG, the lower the opportunity for *Toxoplasma gondii* to proliferate and invade placental tissue.

Ethical approve : Our study was approved by institutional ethical approval company. Following task facilitating request from my organization (College of Medicine, Wasit University) verbal consent was officially obtained from Wasit Health institution all participating women.

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Conflict of Interest : Nil.

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