Evolution ROP16 gene expression of Toxoplasma gondii in pregnant and aborted women

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Abstract
Toxoplasma gondii is an obligate intracellular parasitic protozoan that can cause toxoplasmosis in humans and other vertebrate. *T. gondii* can manipulate the host gene expression by interfering with miRNA expression, which is closely associated with the molecular mechanisms and can induced infection. This study assessed the evolution of ROP16 gene expression in 192 cases that were categorized into 156 pregnant women, and 36 women with abortion. Out of the 156 pregnant women, 23 cases were positive for real time-PCR and out of all 36 aborted women, 16 of them were positive for real time-PCR investigation for *toxoplasma gondii*. The result showed the level of ROP-16 expression was higher significantly in abortion group when compared to pregnancy group, 32 versus 14.83, respectively (p = 0.031). These finding also suggest high expression of ROP 16 gene in blood than placenta tissue.
Keywords: *Toxoplasma gondii*, ROP 16, abortion, pregnant.

1. Introduction

Toxoplasma gondii is an opportunistic food-borne protozoon with an extraordinarily broad host range of all warm-blooded animals, In humans, the most important clinical manifestations are as follows: (i) retinochoroiditis, being the most important cause of posterior uveitis and an important cause of blindness in certain countries (De-la-Torre et al., 2009), (ii) congenital toxoplasmosis, a public health problem responsible for early childhood morbidity and mortality (Gómez-Marin et al., 2011), and (iii) cerebral toxoplasmosis, the most important cause of neurological symptoms in HIV-infected patients (Cardona et al., 2011).
The apical complex of the apicomplexan organisms, consisting of conoid and apical organelles, is responsible for host cell penetration and the establishment of intracellular parasitism. The apical organelles, including rhoptries, micronemes and dense granules, are characteristic secretory vesicles that discharge proteins involved in the cell invasion (Blackman and Bannister, 2001; Dubremetz, 2007; Tagoe et al., 2021). The most extensively studied are ROP16 (TGME49_062730) kinases, phosphorylates STAT3 (Ong et al., 2010) and STAT6 (Yamamoto et al., 2009) transcription factors secreted by rhoptries, this factor is injected into the host cytoplasm during the invasion process, and it translocated into the host cell nucleus (Saeij et al., 2007;Ihara and Nishikawa, 2021).

2. Materials and Methods

Study subjects and case definition

The following study was designed in order to diagnose parasitic virulence factor ROP16 in pregnant and aborted women by using molecular diagnosis Real time PCR method. Specimens were collected aseptically via venous blood sampling of 192 pregnant and aborted women; 100 mg Human placenta tissue samples were transported to a sterile 1.5ml micro centrifuge tube and then stored in -80C for genomic DNA and RNA extracted.

Genomic DNA and RNA Extraction

Genomic DNA was extracted from blood and placenta tissue samples by using (gSYNCTM DNA Extraction Kit / Geneaid Biotech Ltd. Taiwan) and total RNA were extracted from specimens by using (TRIzol® reagent kit) and done according to company instructions. Real Time PCR was performed for detection of T.gondii from blood samples and placenta tissue by using the specific primers and TaqMan probe specific for B1 gene in Toxoplasma gondii this technique was carried out according to method described by (Lin et al., 2000). qPCR master mix was prepared by using (RealMOD™ Probe HP 5X qPCR Mix Kit iNtRON /Korea) and this master mix done according to company instructions. The reactions were done with an AB Step One real-time PCR system (Applied Biosystems) in a final volume of 20µl. the reaction mixture contained 10µl of qReal Master Mix (Amplicon, Denmark), 1 µl of each primer (B1 forward, B1reverse and B1probe primer), PCR water (2µl) and 5µl extracted DNA. The RT-PCR primer that used in gene expression of T.gondii virulence factors genes and housekeeping GAPDH gene were designed in this study by using NCBI Genbank database and perimer3 plus. (Scientific Researcher provided all these primers. Co. Ltd. Iraq) are showed in Table 1.

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Table 1: Nucleotide sequences of real time PCR primer/probe sets			
Primer	Sequence (5'-3')		Product size
B1gene primer	F	TCCCCTCTGCTGGCGAAAAGT	94bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
B1gene probe	FAM-	TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA	
T.gondii rop16 gene	F	TTGATGGCTCCGCATTGAAC	104bp
	R	TTAGGCAGCCACATGCACAAG	
Human GAPDH gene	F	AATTCCATGGCACCGTCAAG	104bp
	R	ATCGCCCCACTTGATTTTGG	

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company. After that, the mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action. DNase-I treated RNA samples were also used in cDNA using M-MLV Reverse Transcriptase kit and done according to company instructions, Than RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice, After that, these qPCR master mix component that mentioned above placed in qPCR plate strip tubes and mixed by Exispin vortex, centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system. After that, the qPCR plate was loaded and the following thermocycler protocol.

3. Result

The present study enrolled 192 cases that were categorized into 156 pregnant women and 36 women with abortion. Out of the 156 pregnant women, 23 cases and out of all 36 aborted women, 16 of them were positive for real time-PCR investigation for T.gondii. Comparison of results of RT-PCR for toxoplasma gondii in blood and placenta between abortion group and pregnancy group is shown in table 1

Table 1: Comparison of results of RT-PCR for toxoplasma gondii in blood and placenta between abortion group and pregnancy group			
RT-PCR	Abortion	Pregnancy	P
	n = 36	n = 156	
Blood RT-PCR toxoplasma gondii			
Positive, n (%)	9 (25.0 %)	23 (14.7 %)	0.137 C
Negative, n (%)	27 (75.0 %)	133 (85.3 %)	NS
Placenta RT-PCR toxoplasma gondii			
Positive, n (%)	7 (19.4 %)		
Negative, n (%)	29 (80.6 %)		
n: number of cases; **C**: chi-square test; **NS**: not significant; *: significant at $p \leq 0.05$			

The level of ROP-16 expression was higher significantly in abortion group when compared to pregnancy group, and ROP-16 expression was higher significantly in blood comparison with placenta were show in table 2 and 3 respectively.

Table 2: Comparison of median blood ROP-16 expression fold change between abortion group and pregnancy group				
Characteristic	Abortion group	Pregnancy Group	*P Mann Whitney U test*	interpretation
Blood ROP16 expression				
Median	32	14.83	0.031	Significant

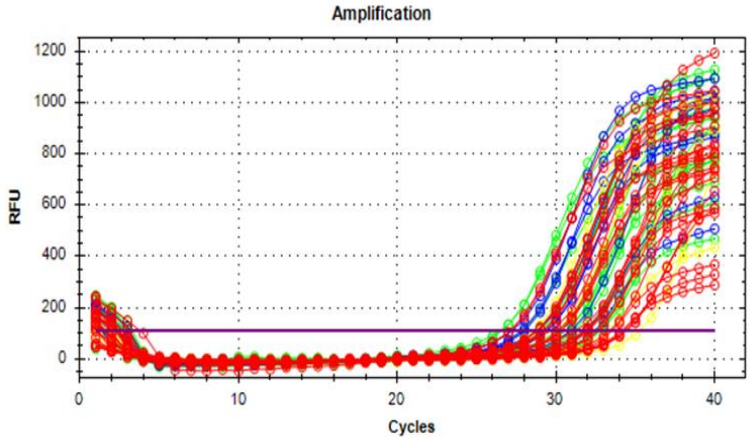

Fig 4: Real Time PCR amplification plot for rop16 gene in Toxoplasma gondii positive samples. Green plots (Aborted woman group samples), yellow plots (Patient placenta group samples), Blue plots (Patient Blood group samples), and the Red plots (Patient pregnant group samples).

Table 3: Comparison of median ROP-16 fold change expression between placenta and blood in abortion group.

Characteristic	Placenta	Blood	p Wilcoxon test	Interpretation
Median	14.72	32.00	0.017	Significant

4. Discussion

Toxoplasma gondii is a widespread parasite that infects warm-blooded vertebrates, this protozoan can cause serious illness, especially in immunocompromised patients and in pregnant women, maternal acute toxoplasmosis during pregnancy is one of the important factors that increase the chance of abortion. (Kheirandish et al., 2019; Mocanu et al., 2022). Real-time PCR which is the best-performing accurate technique till now that has been described as a modern high sensitive assay for early detection of minimal amounts of parasitic DNA in different types of samples such as blood and placental tissue (Mousavi et al., 2018). in study by Khademi et al., (2022) the parasite's DNA was detected in 18% (9/50) and 14% (7/50) of blood and placenta samples, respectively these finding agreed with us, and incompatible with Shaker et al., (2018) result showed 15/100 (15%): 6/100(14.3) pregnant and 9/100 (15.5%) aborted women were positive by RT-PCR for toxoplasmosis.

In Wu et al., (2020) study, to estimation the level of ROP-16, it was higher significantly in blood comparison with placenta, 32 versus 14.72, respectively ($p = 0.017$). Jensen et al., (2013) and Chen et al., (2020) study ROP16 phosphorylates driving macrophages toward an alternatively increased IL-10 production and a Th2 polarized response during infections and pregnancy or abortion. Indicated that because Toxoplasma-induced placental inflammation is associated with early abortions, it is likely that Toxoplasma effectors inducing a proinflammatory response play an important role in the pathology of such cases (Wang et al. 2018)

On the other hand, Cheng et al., (2015) revealed that Toxoplasma strains carry ROP16 effectors in placenta was different and more active than in blood from those of the archetypal types; therefore having an opposing inflammation-inducing feature. It is possible that other parasite effectors might be responsible for the biased immunity in the placenta; however, these studies support the notion ROP16 may play an important role in the pathogenesis of abortion. Cui et al., (2020) also reported that a Rop16 strain, which lacks an active form, was assessed in pregnant mice. Infection with the rop16 strain produced higher virulences, with increased TNF-α and IL-12 levels and decreased TGF-β and may resulted in abortion state. Following the invasion of the host cell by T. gondii, ROP16 localizes to the nucleus and phosphorylates STAT3, leading to prolonged stimulation of this transcription factor and activation of STAT3- dependent stimulation promoters in blood (Yamamoto et al., 2009). Whether these or similar molecular adaptations are activated in mammalian host cells following infection by T. gondii and release of ROP16 remains a relevant question to be answered by future studies (Li et al., 2016).

5. Conclusion

T. gondii is the most commonly occurring intracellular protozoan parasite in the world. Despite numerous studies having been conducted on toxoplasmosis over many years, the prevalence of *T. gondii* in humans remains unacceptably high. Potentially virulent from many different countries remain poorly studied and are thus not elucidated clearly. A deeper understanding of the virulence mechanisms and pathophysiology of this obligate intracellular pathogen will not only improve diagnosis but is an essential prerequisite to successful vaccine design and drug discovery.

Reference

De-la-Torre, A.; López-Castillo, C. A. and Gómez-Marín, J. E. (2009). Incidence and clinical characteristics in a Colombian cohort of ocular toxoplasmosis. *Eye*, *23*(5), 1090-1093.

Blackman, M. J. and Bannister, L. H. (2001). Apical organelles of Apicomplexa: biology and isolation by subcellular fractionation. *Molecular and biochemical parasitology*, *117*(1), 11-25.

Cardona, N.; Basto, N.; Parra, B.; Zea, A. F.; Pardo, C. A.; Bonelo, A. and Gómez-Marin, J. E. (2011). Detection of *Toxoplasma* DNA in the peripheral blood of HIV-positive patients with neuro-opportunistic infections by a real-time PCR assay. *Journal of Neuroinfectious Diseases*, *2*.

Chen, L.; Christian, D. A.; Kochanowsky, J. A.; Phan, A. T.; Clark, J. T.; Wang, S. and Hunter, C. A. (2020). The *Toxoplasma gondii* virulence factor ROP16 acts in cis and trans and suppresses T cell responses. *Journal of Experimental Medicine*, *217*(3).

Cheng, W.; Liu, F.; Li, M.; Hu, X.; Chen, H.; Pappoe, F. and Shen, J. (2015). Variation detection based on next-generation sequencing of type Chinese 1 strains of *Toxoplasma gondii* with different virulence from China. *BMC genomics*, *16*(1), 1-9.

Cui, W.; Wang, C.; Luo, Q.; Xing, T.; Shen, J. and Wang, W. (2020). *Toxoplasma gondii* ROP16I deletion: The exacerbated impact on adverse pregnant outcomes in mice. *Frontiers in Microbiology*, *10*, 3151.

Dubremetz, J. F. (2007). Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. *Cellular microbiology*, *9*(4), 841-848.

Gómez-Marin, J. E.; de-la-Torre, A.; Angel-Muller, E.; Rubio, J.; Arenas, J.; Osorio, E. and Castaño, G. (2011). First Colombian multicentric newborn

screening for congenital toxoplasmosis. *PLoS neglected tropical diseases*, *5*(5), e1195.
Hamie, M. (2019). *Insights towards a better understanding and novel treatment modalities of Toxoplasmosis* (Doctoral dissertation, Université Montpellier).
Ihara, F. and Nishikawa, Y. (2021). *Toxoplasma gondii* manipulates host cell signaling pathways via its secreted effector molecules. *Parasitology International*, *83*, 102368.
Jensen, K.D.C.; Hu, K.; Whitmarsh, R.J.; Hassan, M.A.; Julien, L.; Lu, D.; Chen, L.; Hunter, C. A. and Saeij, J.P.J. (2013). *Toxoplasma gondii* rhoptry 16 kinase promotes host resistance to oral infection and intestinal inflammation only in the context of the dense granule protein GRA15. *Infection and immunity, 81(6),* 2156–2167. [Google Scholar] [CrossRef][Green Version].
Khademi, S. Z.; Ghaffarifar, F.; Dalimi, A.; Davoodian, P. and Abdoli, A. (2022). Spontaneous abortion among *Toxoplasma gondii* IgG seropositive women: Molecular detection, genotype identification, and serological assessment with conventional ELISA and avidity ELISA. *Journal of Obstetrics and Gynaecology Research*, *48*(10), 2479-2485.
Kheirandish, F., Ezatpour, B., Fallahi, S., Tarahi, M. J., Hosseini, P., Rouzbahani, A. K. and Akbari, S. (2019). *Toxoplasma* serology status and risk of miscarriage, a case-control study among women with a history of spontaneous abortion. *International Journal of Fertility & Sterility*, *13*(3), 184.
Li, X. Z.; Lv, L.; Zhang, X.; Anchang, K. Y.; Abdullahi, A. Y.; Tu, L. and Yuan, Z. G. (2016). Recombinant canine adenovirus type-2 expressing TgROP16 provides partial protection against acute *Toxoplasma gondii* infection in mice. *Infection, Genetics and Evolution*, *45*, 447-453.
Lin, M. H.; Chen, T. C.; Kuo, T. T.; Tseng, C. C. and Tseng, C. P. (2000). Real-time PCR for quantitative detection of *Toxoplasma gondii*. *Journal of clinical microbiology*, *38*(11), 4121-4125.
Mahmood, A. R.; Abdulla, A. K. and Hussein, N. M. (2021). Molecular detection of *Toxoplasma gondii* specific repeat element in blood of recurrent aborted women by real-time PCR. *Periodicals of Engineering and Natural Sciences*, *9*(4), 708-714.
Mocanu, A. G.; Stoian, D. L.; Craciunescu, E. L.; Ciohat, I. M.; Motofelea, A. C.; Navolan, D. B. and Craina, M. (2022). The Impact of Latent *Toxoplasma gondii* Infection on Spontaneous Abortion History and Pregnancy Outcomes: A Large-Scale Study. *Microorganisms*, *10*(10), 1944.
Mousavi, P.; Mirhendi, H.; Mohebali, M.; Shojaee, S.; Valian, H. K.; Fallahi, S. and Mamishi, S. (2018). Detection of *Toxoplasma gondii* in acute and chronic phases of infection in immunocompromised patients and pregnant women with real-time PCR assay using TaqMan fluorescent probe. *Iranian Journal of Parasitology*, *13*(3), 373.
Negero, J.; Yohannes, M.; Woldemichael, K. and Tegegne, D. (2017). Seroprevalence and potential risk factors of *T. gondii* infection in pregnant women attending antenatal care at Bonga Hospital, Southwestern Ethiopia. *International Journal of Infectious Diseases*, *57*, 44-49.
Ong, Y. C., Reese M. L., and Boothroyd, J. C. (2010). *Toxoplasma* rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6. *Journal of Biological Chemistry*, *285*(37), 28731-28740.
Saeij, J. P. J.; Coller, S.; Boyle, J. P.; Jerome, M. E.; White, M. W., and Boothroyd, J. C. (2007). *Toxoplasma* co-opts host gene expression by injection of a polymorphic kinase homologue. *Nature*, *445*(7125), 324-327.
Shaker, M. J.; Darweesh, N. H.; Hussein, R. A. and Salman, S. T. (2018). Immunological and Molecular study of *Toxoplasma gondii* from aborted women in Diyala/Iraq.
Tagoe, D. N., Drozda, A. A., Falco, J. A., Bechtel, T. J., Weerapana, E., and Gubbels, M. J. (2021). Ferlins and TgDOC2 in *Toxoplasma* microneme, rhoptry and dense granule secretion. *Life*, *11*(3), 217.
Wang, C.; Cheng, W.; Yu, Q.; Xing, T.; Chen, S.; Liu, L. and Xu, Y. (2018). *Toxoplasma* Chinese 1 Strain of WH3Δ rop16I/III/gra15II Genetic Background Contributes to Abnormal Pregnant Outcomes in Murine Model. *Frontiers in immunology*, *9*, 1222.
Wu, L.; Wu, L.; Xi, C.; Liu, Y.; Jiang, X.; Chen, S. and Cao, J. (2020). *Toxoplasma gondii* rhoptry protein 16 (ROP16) modifies apoptosis in human 293T cells. *Journal of Nanoscience and Nanotechnology*, *20*(1), 24-30.
Yamamoto, M.; Standley, D. M.; Takashima, S.; Saiga, H.; Okuyama, M.; Kayama, H., and Takeda, K. (2009). A single polymorphic amino acid on *Toxoplasma gondii* kinase ROP16 determines the direct and strain-specific activation of Stat3. *Journal of Experimental Medicine*, *206*(12), 2747-2760.