Detection of *Toxoplasma gondii* genotypes in abortion women by RFLP-PCR in Al-Najaf Al-Ashraf province

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**Abstract.** The study conducted on 400 aborted women and 30 healthy women as control groups. All these cases were defined as suspected with *Toxoplasma gondii* and examined by measuring IgG & IgM serum level when attended to Public Health Laboratory, in An-Najaf province from November 2015 to May 2016. seventy eight out of 400 (19.5%) women were found infected with *T. gondii* by this test, only 23 positive samples were used in polymerase chain reaction – restriction fragment length polymorphism for detection genotypes of *T. gondii* strains By use three restriction enzymes MseI to nested PCR products GRA6 and BsiE1 & TaqI to nested PCR BTUB products, to detected strain types of *Toxoplasma gondii*. The results are : 13 of type I (60%), 6 of type II (25 %), and 4 of type III (15%). This can provide possible additional studies of high importance, as they will provide better understanding of the different strains of parasite genotypes is present in samples and how different genotypes are circulating nature. All the evidence provided might be helpful for showing the association between strains type of *T. gondii* and toxoplasmosis in infected women and aid in providing preventive actions.

1. **Introduction**

*Toxoplasma gondii* is an obligate intracellular parasite which is capable of infecting warm-blood animal and human. Livestock is known as the important source of infection for human [1]. Human could be infected by ingestion of undercooked meat containing bradyzoite form in tissue cyst or water contaminated with oocyst shed by infected cats [2]. However, it is well indicated that the complications and severity of toxoplasmosis depend on the immunological condition of individuals; recent investigations have revealed that the genetic of parasite plays an important role [3].

The most common pregnancy complication is fetal loss, occurring in 25-30% of recognized pregnancies. Recurrent pregnancy loss affects at least 1% of all couples [4] and can be defined as two or more failed pregnancies [5]. *T. gondii* is one of the important obligate intracellular protozoan parasites, classified in the phylum Apicomplexa, a pathogen in veterinary and human. It is enters the host via the digestive system and poses a severe risk for congenitally infected infants [6]. There are three types' strains of *T. gondii*. Type 1, 2 and 3 strains, type 1 is highly virulent [7].

The diagnosis of recent infection of toxoplasmosis has been characterized either by expressing a specific immunoglobulin (IgM) antibody, a noticeable increase in specific IgG antibodies, or both. Study of toxoplasmosis is spurred for essentially three Causes. First, Toxoplasma can cause dangerous diseases, e.g. encephalitis, retinitis, myocarditis and pneumonia [8]. Second, Toxoplasma is used as model-system of Apicomplexa parasites [9]. Toxoplasma is an important veterinary pathogen with high estimated costs owing to disease, abortion or vaccination in animal farming [10].

Today the polymerase chain reaction (PCR) used to identification nucleic acid of *T. gondii* in clinical samples, technique of PCR performed by identification of the parasite DNA directly, and the findings independent of the immunologic states of the patients. [11, 12, 13, 14]

The PCR technique has been refined, and can be detected of low amounts of the parasite DNA. The PCR sensitivity and specificity depend upon several factors as purity and amount of extracted DNA, the DNA sequence characteristic, and optimum condition of the reaction. The principal problem with PCR used to detect *T. gondii* is the absences a standardized protocol [15]. Three different methods of PCR principles for identification of *T. gondii* DNA are described below; PCR oligochromatography, real-time PCR and conventional PCR.
Molecular assays, such as the PCR make it possible to detect small quantities of target DNA and potentially provide an alternative sensitive diagnostic tool [16]. The important marker used in recognized between three genotypes of T. gondii was GRA6 gene [17, 18].

2. Material and methods

2.1. Genotyping isolation DNA by RFLP-PCR.

All of procedures and the experiments in this study were approved and validated by the Ethical Committee at the University of Kufa Faculty of Science and the samples were collected from Public Health Laboratory in An-Najaf province. Seventy eight out of 400 (19.5 %) women were found infected with T. gondii by this test; only 23 positive samples were used in RFLP-PCR for detection genotypes of T. gondii strains by used three markers BTUB, GRA6 and GRA7. Genotyping of the 23 samples as the following results: 13 genotype I (60%), 6 genotype II (25 %) and 4 genotype III (15%).

A total of 23 clinical samples as abortion case (designated KU01 through KU23) were collected during the period of November 2015 to Jun. 2016. DNA extraction and isolated from abortion tissues and blood by using genomic DNA purification kits , Wizard and Dext (Promega, Madison, Wis.) with the manufacturer’s instructions.

Identifying of Toxoplasma. gondii by PCR assay , infections of it at first were confirmed by nested PCR amplification of the conserved and repetitive genes BTUB, GRA6 and GRA7 which are located on chromosome IX, chromosome X and chromosome VII respectively . We achieved (nPCR) as a highly sensitive markers from three different genetic: BTUB, GRA6 and GRA7 which are located on chromosomes IX, X and VII respectively, These markers conceder as a highly sensitive markers, and the presence of polymorphism sequence in every locus in restriction fragment length polymorphism(RFLP) were used as an indicator to assign alleles for genotypes strain.

The PCR mix for nested was could to carry in a 50ul reaction mixing, consisted of 2ul of DNA template, 1.5 ul of R&F primer (50uM), 10 ul of 2X master mix (AccuPower® PCR PreMix from Bioneer) 10 x of PCR buffer in a final volume of 50 ul. The first step of PCRs were performed using the primer pairs GRA7. F / GRA7. R, GRA6. F / GRA6. R and Btb. F / Btb. R. Then for second round amplification (nested PCRs) used (5 ul) of PCR amplified products of each marker mixed in a 25 ul of reaction mixture (as given above) by using primers GRA7.F /GRA7. R, GRA6.F/ GRA6.R and Btb F / Btb R. respectively. PCR products were resolved in a 1.5% agarose gel stained with ethidium bromide. Each reaction was done in at least three independent replicates. Primer sequences and thermos cycling parameters are detailed in Tables 1. Negative controls consisted of all components were at the PCR reaction excluding DNA.

The amplified fragments were digested with appropriate restriction enzymes for GRA6 by Msel enzyme where incubated at 37°C for 4-5 hours and for BTUB by BsiE1 & TaqI enzyme by incubated it at 60°C for one hour. Msel enzyme could cut the nested PCR product in different fragment for Type I into tow fragments of 544 bp & 194 bp , while Type II into 700bp and 100bp and for Type III strain to 600bp and 100bp. Digestion of the amplified product of BTUB with TaqI were distinguished genotype II from genotypes I and III and with BsiE1 distinguished genotype I (allele 1) from genotypes II and III . The fragments of all products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and the bands were identified under UV transilluminator (Cleaver Scientific, UK. ) Estimates of the sizes of fragments were based on comparison to _X 174 DNA digested with BsiE1&TaqIfor BTUB and Msel for GRA6 (New England Biolabs, NEB #B7025)

| Gene  | Chromosome | Primer sequences | TA. Base bare | Retraction Enzyme |
|-------|------------|-----------------|---------------|-------------------|
|       |            |                 |               |                   |

Table1. Primers of Toxoplasma gondii of nested PCR.
3. Results

By the use highly sensitive genotyping of *T. gondii*, with three markers BTUB, GRA6 and GRA7, by nPCR. 23 samples were collected from 23 patients (23 clinical samples as abortion case) were all it analyzed and examined to identify the presence of *T. gondii* genotype in samples. All of the clinical samples were confirmed for the presence of the parasite by nested PCR as positive In GRA7 and GRA6 while in BTUB *T. gondii* gene excluding sample KU20 and KU21 were absent. In first step in GRA7 gene, samples showed a band in 322bp and after the second step the internal primer of the nested-PCR showed a band in 222 bp. While in negative control was absent show (figure1). In GRA6 were showed a 950 bp in first step and in 750 bp in nested PCR step (figure2). In BTUB the external primer were showed 500 bp bands and 411 bp after second step (fig. 3). .Genotyping of the 23 samples achieved results as the following: 13 of genotype I (60%), 6 of genotype II (25 %), and 4 of genotype III (15%).

The PCR assay was used the three genotypes strain. Genotype I RH strain, Genotype II strain Beverly and Me 49, and the Genotype III was strain C56CTG (1), were distinguished the three types by RFLP- PCR assay. The amplified nested PCR product of BTUB gene was digested with restriction enzyme BsiEI when incubated it at 60°C for 1 hours, distinguished genotype I (allele 1) from genotypes II and Type III(allele 2) while with restriction enzyme TaqI is clearly distinguished genotype II (allele 2) from genotypes I and Type III (allele 1) (Fig. 4).

Msel enzyme could cut the nested PCR product of GRA6 gene in different fragment. For genotype I into tow fragments of 544 bp & 194 bp , while genotype II into 700bp and 100bp and for genotype III to 600bp and 100bp (Fig. 5). The results of the gene GRA6 RFLP-PCR showed that the number of genotype I, genotype II and genotype III were 13, 6 and 4 respectively. Consequently, we can detect all three genotypes of *T.gondii* by two markers based on distinct assay RFLP PCR patterns.

Table 1. Represent the numbers of genotyping strain of *T. gondii* clinical samples as abortion case KU01 toKU23 and there Percentage.

| Type of strain | The sample                  | Number of samples | Percentage 100% |
|----------------|-----------------------------|-------------------|-----------------|
| I              | KU01, KU03, KU04, KU06, KU07, KU08, KU09, KU10, KU13, KU17, KU18, KU20, KU22 | 13                | 56.52%          |
| II             | KU05, KU12, KU14, KU16, KU19, KU23                                   | 6                 | 38.333%         |
Figure 1. 2% agarose gels stained with ethidium bromide showing Nested PCR amplification of gene GRA7 of *Toxoplasma gondii* from clinical samples as abortion case KU01 to KU10 and Negative controls (N.C.) consisted of all components were at the PCR reaction excluding DNA. Lanes M, molecular mass markers ladder 100bp (New England Biolabs Inc.). The left picture (1) was the first step and the right (2) was second step nested PCR, 322bp and 222bp respectively was indicate to the expected amplification of each reaction.

Figure 2. 2% agarose gels stained with ethidium bromide showing Nested PCR amplification of gene GRA6 of *T. gondii* from clinical samples as abortion case KU01 to KU10 and Negative controls (N.C.) consisted of all components were at the PCR reaction excluding DNA. Lanes M, molecular mass markers ladder 100bp (New England Biolabs Inc.) The left (1) picture was the first step and the right (2) was second step nested PCR, 950 bp and 750 bp respectively was indicate to the expected amplification of each reaction.

Figure 3. 2% agarose gels stained with ethidium bromide showing Nested PCR amplification of gene BTUT of *T. gondii* from clinical samples as abortion case KU01 to KU10 and Negative controls (N.C.) consisted of all components were at the PCR reaction excluding DNA. Lanes M, molecular mass markers ladder 100bp (New England Biolabs Inc.). The left (1) picture was the first step and the right (2)
was second step nested PCR, 500 bp and 411 bp respectively was indicate to the expected amplification of each reaction.

![Figure 4](image_url)

**Figure 4.** 2% agarose gels stained with ethidium bromide showing RFLP-PCR analysis of genotypes strains of *Toxoplasma gondii* (sample KU02). (A). Digestion of of BTUB with TaqI enzyme which distinguished type II from type I and type III and (B). Enzyme BsiEI which distinguished type I from type II and type III

![Figure 5](image_url)

**Figure 5.** 2% agarose gels stained with ethidium bromide showing RFLP-PCR analysis of genotypes strains of *Toxoplasma gondii* (sample KU02). Digestion of GRA6 with MseI enzyme which distinguished three types by the length of DNA fragments where cutting genotype I into two fragments 544bp and 194 bp band while cutting genotype II into 2 fragments 700bp and 100bp, and the genotype III could cut it into tow fragments 600bp and 100bp as showing above from type I and type III.

4. Discussion

The *T. gondii* is infected one-third of the human world population [22]. There are different strains and type of *T. gondii* associated with the human toxoplasmosis. In this study we used a RFLP PCR protocol to determining the genotype of *T. gondii* strains isolated from abortion blood and tissues. Nested PCR amplification of the GRA7, GRA6 and BTUB treated by RFLP analysis, assigned all samples to one of three specific lineages of *T. gondii*. Type II strains of *T. gondii* were most of these samples, thus presenting further evidence that strains of this genotype cause the majority of toxoplasmosis in humans [23], while in present study we found the genotype I strains of *Toxoplasma gondii* were most frequencies of the samples (60%) in contrast Type II were 25% and the strain type III just 15%, however our data showing that a differences is not so high, in particular if it is considered that just 23 sample individuals in our study. Most investigators have used the GRA7, GRA6 and BTUB gene for detection of strains types of *T. gondii* however, these loci are not very sufficiently polymorphic to allow strain typing [24].
We choose GRA7 gene as the marker since it has a large number of sequences, which can providing a significant representation of the isolates. The GRA7 gene presents only in *T. gondii* and in the protozoon *Hammondia hammondi*, this protozoon doesn’t infect a human [25].

GRA7 gene is a suitable target for primer design. Nested-PCR assay is considered as a hopeful solution to identify a wide range of strains of *T. gondii* [21]. So in this study we used this marker in nested-PCR assay to determining the genetic diagnosis of *T. gondii*, while we used Nested RFLP-PCR method to employ two genetic markers, GRA6 and BTUB genes for detection of strains types of *T. gondii*. Several technical challenge were found in current study for example when using multi locus typing, almost the samples were limited in volume and in DNA amounts in abortion tissues and blood. The purpose of present study is to identify the sensitive nested PCR assay and RFLP PCR protocol to detect the genotype strain of *T. gondii* in women abortion. Nested-RFLP-PCR assay as a good proof that may supports fast and easy choice of diagnostic of *T. gondii* and to give us a perfect prevention of animals (human) that are of pathogens affects [26,27].

The differential of *T. gondii* genotypes in our data which shows all three types strains from aborted tissue and blood women in al-Najaf province there are several possible explication for that variation which observed in *T. gondii* genotypes may be was a different geographic regions of patients in the al-Najaf province and the epidemiological prevalence of the *T. gondii* and may be the different of the rout of transmission in the regions of the study that’s lead to variation of effect of differential of strain selection [28].

**5. Conclusion**

In conclusion, Nested polymerase chain reaction –restriction fragment length polymorphism assay which is described in the present study is highly sensitive and able to detect few parasites in samples test that also contain mammalian genomes. Consequently, the above protocol can be applied to possible to primary clinical samples to detect and diagnose toxoplasmosis. Nested polymerase chain reaction – restriction fragment length polymorphism analysis is advantageous in providing rapid, unambiguous assignment of a parasite genotype by restriction fragment length polymorphism analysis. This can provide possible additional studies of high importance, as they will provide better understanding of the different strains of parasite genotypes is present in samples and how different genotypes are circulating nature. All the evidence provided might be helpful for showing the association between strains type of *T. gondii* and toxoplasmosis in infected women and aid in providing preventive actions.

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