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A Comparative Study of The Diagnosis of *Toxoplasma Gondii* in Human Placenta by Traditional Method, Restriction Fragment Length Polymorphism, and The Immunohistochemistry Method

Saafa Ressan Abdullah Al-Kaeebi [1*], Ghaidaa Abass Jasim Al- Jubouri [2]

[1] College of Nursing, Al-Qadisiya University, Iraq.

[2] College of Veterinary Medicine, Al-Qadisiya University, Iraq.

*** Corresponding author:** Saafa Ressan Abdullah Al-Kaeebi, **Mobile: 009647801827487 Email:** safaa.abdullah@qu.edu.iq

ABSTRACT

Introduction: *Toxoplasma gondii* is an animal source of great importance in veterinary and public health.

Objective: The aim of this study was to detect genotyping of *T. gondii* by PCR-RFLP and to detect infection in placental tissue by immunohistochemistry (IHC) and to determine whether there is a correlation between the results obtained by this method and those obtained by through the traditional Impression method.

Material and methods: A total of 100O samples of placenta were selected for women who had aborted in the first months of pregnancy. In the beginning, all samples were stained with Gimsa stains using the steps of the Impression method, in which 40 (40%) samples were diagnosed with Toxoplasma infection, and 60 (60%) samples were not infected with Toxoplasma. The GRA6 gene was then amplified using the PCR method for all 40 positive tissue samples. In addition, an MseI exonuclease (PCR-RFLP) was added to all PCR products resulting from the amplification of the gene.

Results: After performing PCR-RFLP, it was discovered that the number of samples belonging to type I is 19, 13 for type II, and 2 for type III. The percentages were 47.5%, 32.5%, and 5% for the 3 types, respectively. Only 4 unknown and 2 negative samples were recorded. As for using the tissue immunohistochemistry method, and after tissue cutting of the 40 samples and the use of DAB, the result was 38 samples infected with toxoplasma plasma, which constituted 95%, while 2 samples were not infected with a percentage of 5%.

Conclusion: Several techniques can be used, but the immunohistochemistry technique is considered as a confirmatory test to classify aborted women into *T. gondii* infected women and non *T. gondii* infected women, and that PCR -RFLB method enables the identification of the most important species of *Toxoplasma* widespread.

Keywords: Toxoplasma gondii, Impression method, PCR-RFLP, immunohistochemistry, Experimental study, Al-Qadisiya University.

INTRODUCTION

Toxoplasma gondii is a single parasite caused Toxoplasmosis. This parasite is an intracellular binding parasite, commonly known as toxoplasmosis; it causes toxoplasmosis, a common disease among others. In humans and animals, in addition to being spread among individuals suffering from immunodeficiency, while immune healthy people are asymptomatic, the incidence ranged from less than 10% to about 90% depending on the diagnosis of parasite antibody counters [(1)]. The parasite is characterized by its ability to invade all nucleated cells and multiply inside them and move to various organs in the host's body, leading to the spread of infection in the body, which indicates the parasite's ability to penetrate different life barriers such as the placenta [(2)]. From pregnancy, it leads to various congenital malformations of the fetus and this may lead to miscarriage [(3)].

Due to the fact that the clinical signs resulting from toxoplasmosis infection are not clear, so they cannot be relied upon for diagnostic purposes. Therefore, laboratory diagnosis is the main method for detecting the infection. There are many laboratory diagnostic methods that have been used in research and studies [(4,5)]. Impression smear, isolation of the parasite, serological tests, genomic method, and immunohistochemistry detection [(6)].

Epidemiological studies have identified the most important and dangerous modes of transmission of the parasite: owning cats, eating raw or unwashed fruits and vegetables, eating raw or undercooked lamb, beef and minced meat products, animal husbandry, and handling soil [(7,8)]. Serological methods depend on sensitivity and specificity (based on the complex immune mechanism) including affinity [(9)]. The LAT test is the standard test for Toxoplasma antibody detection worldwide, immunofluorescence tests (lFAT) and rarely by ELISA [(10,11)]. Immunohistochemistry can detect antigens in cells of a tissue section depending on the principle of specificity of the binding of antibodies specifically to antigens in biological tissues [(12)].

MATERIALS AND METHODS

Sample collection. The study samples were collected from the Maternity and Children Hospital in Al-Diwaniyah and Al-Furat Private Hospital, where 100 placentas were collected from aborted women during the first months of pregnancy in the maternity halls of these hospitals.

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Diagnosis of Parasite

Impression Smear. The parasite was diagnosed using Impression Smear method to confirm its presence in placenta samples, which was adopted as a primary diagnostic method by observing the presence of parasite stages, and samples were prepared by cutting part of the placenta and making a smear. On a clean glass slide from the cut side and along the glass slide, then air-dried, fixed with 90% methyl alcohol for ten minutes, stained with Gimza stain for 15 minutes and examined under a microscope to see the parasite [13]. A portion of the positive samples were isolated for the purpose of PCR - RFLB testing and the GRA6 gene was used for the purpose of genotyping. Also, a part of the same infected placenta was cut and placed in formalin at a concentration of 10% for the purpose of tissue section and the method of immunohistochemistry.

Genomic method Restriction Fragment Length Polymorphism (RFLP)

DNA extraction. After confirming the 40 positive samples for infection in the first method, the company's method (Promega/ USA) was used, and 20 mg of placenta was cut and instructions were followed to obtain the final product of DNA.

Agarose gel electrophoresis of DNA. The electrophoresis has been performed to determine the quality of the DNA extractions and to visualize the PCR product size after finishing the PCR programme. For DNA quality the agarose gel was 0.7%, while it was 1-2% for the regular PCR products.

RFLP-PCR

1. The preparation of agarose gel was performed according to the protocol that was previously described [14].
2. **Loading the PCR products.** About 5 µl of each PCR products were inserted into the middle of it correspondence hole, About 5 µl of Safe-Green 100bp Opti-DNA Marker was added to served as a marker for measuring the size of the PCR products.
3. **Electrophoresis.** Following the loading of Safe-Green 100bp Opti-DNA Marker and the samples. Finally, the gel was transferred into UVP system to fisualise the PCR products under 320nm UV light source.
4. **Preparation of Primers.** According to instruction of the primer synthesiser company, concentration of 10 µM/µl was prepared from the stock primers to be used as a work primer [15]. Extracted DNA was amplified with the GRA6 gene primer. Primer pairs consist of a GRA forward primer (GTAGCGTGCTTGTTGGCGAC) and a reverse GRA primer (TACAAGACATAGAGTGCCCC). Approximately 773 bp can be amplified by these primers. PCR was performed in a ready mix (GoTaq® G2 Green Master Mix/promega/USA) final mixture.
5. **PCR master mix.** According to the company's instructions reaction mixture was prepared GoTaq® G2 Green Master Mix (1X ,25 µl), Forward primer and Reverse primer (10 µM/µl, 4 µl) for each one, ddH_2O (13 µl), and DNA (40 ng, 4 µl), then put into PCR Thermocycler T100 Thermal cycler. BioRad USA. According to specialized conditions, initial denaturation (94°C, 5 min, 1X), Denaturation (94°C, 30 sec, 35X), Annealing (56°C, 30 sec, 1X), Extension (72°C, 1 min, 35X), Extension (94°C, 5 min, 1X), and Final extension (72°C, 5 min, 1X).
6. **Restriction Fragment Length Polymorphism (RFLP).** PCR-RFLP was performed on PCR products of positive samples to determine the parasite strain, and the use of MseI exonuclease (Cut Site-TTAA, Concentration -10.000 unit/ml, Buffer-CutSmart, USA) was used to digest the products. This restriction enzyme characterizes different types of Toxoplasma by slicing the products into 168 and 544 bp for type I, 75 and 623 bp for type II, and 97 and 544 bp for type III [15]. After adding the enzyme, the mixture of the product and enzyme mixture is incubated at 37 °C for 4 hours. Finally, staining and examination under UV light were performed to determine the genetic type of the parasite strain.

Histological Sections. The tissue sections were prepared from the organs included in the study by following the steps described as follows [16]:

1. **Fixation.** Samples were fixed immediately after dissection in formalin solution (10%), the appropriate time for fixation ranged between 24-48 hours.
2. **Washing.** Samples were washed after fixation with running water for half an hour to remove all fixative residues.
3. **Dehydration.** Removing the water completely from the tissue by transferring the samples to a series of gradually rising concentration ethyl alcohol using a tissue scroll device Histokinete, which are: 50%, 70%, 80%, 90%, 95% and 100% and twice for the last two concentrations for a period ranging from (1 to 2) hours for each Concentration.
4. **Clearing**. It is apply to remove the dehydrant agent from the specimens and replace it with solutions mixed with molten paraffin wax. Xylene was used and repeated twice for a period ranging from half an hour to an hour.
5. **Infiltration.** Place the samples in melted paraffin wax at a temperature of 56-58 °C and repeat it twice for a period of one to two hours in a convection oven at ranging temperature between 58-60 °C.

6. **Embedding.** The samples were transferred to molds of plastics containing melted paraffin at 56-58 °C. The samples were placed appropriately and the molds were left at room temperature to solidify and frozen until cutting time.

Immunohistochemistry detection

The method of detection is as follows and manufacturer instructions [17,18].

1. **Sectioning.** Paraffin embedded placenta tissue sample were taken to the microtome for sectioned at 4 µm thickness and placed carefully in water bath using a hot plate and mounted on positive charged glass slides (Santa cruz biotechnology, USA).
2. **Deparaffinized.** Pre-heated The placenta tissue section in oven at 55°, then in two changes (10 minutes each) of xylene for deparaffinized, and put for two minutes at four each flow change of 100%, 100%, 90% and 70% ethanol, respectively. Rinse the tissue sections in distilled water and submerge them in Tris-buffered saline (TBS) bath (EnVision FLEX Wash Buffer, SM831) bath for 5 minutes.
3. **Antigen retrieval.** In a glass jar filled with antigen retrieval solution (EnVision™ FLEX Target Retrieval Solution, High pH, DM828) placed The tissue sections , then heated at 60° in a water bath and incubated when water bath temperature arrive at 97° for 25 minutes cool. The tissue sections at room temperature in a glass jar for 20 minutes, then rinsed with distilled water and immersed in TBS buffer bath for 5 minutes.
4. **Gene Tech Pen.** The excessive buffer was removed by wiped gently with a tissue paper around the tissue section, then by a special wax pen (Gene Tech Pen, China). The tissue sections were circled with wax on the glass slides to ensure the reagent was confined only on the tissue section of the slide.
5. **Blocking**. After rinsed the tissue sections immersed in two change of buffer bath (EnVision FLEX Wash Buffer) for 5 minutes each, then removed. The excessive buffer by wiped gently with a tissue paper around the sections flooded. The tissue sections in 100 µL of a blocking reagent (peroxidase block solution) and incubated for 10 minutes in a humidity chamber, removed. The excess buffer on the tissue sections by wiped gently with a tissue paper around the tissue sections. Sections were rinsed and immersed for 5 min in tow changes of buffer bath.
6. **Primary antibody.** Anti-*Toxoplasma gondii* primary Antibody (Polyclonal Rabbit Antibody: MBS373041) was used for detection *T. gondii* in placental tissue in current study .Antibody is diluted 100-fold with antibody diluent (EnVision FLEX Antibody Diluent, Dako, K8006, Denmark). Tissue sections incubated with 100 µL of diluted anti-Toxoplasma gondii primary antibody in a humidity chamber at room temperature for 1 hour, Sections were rinsed and immersed for 5 min in tow changes of buffer bath (EnVision FLEX Wash Buffer) removed. The excess buffer on the tissue sections by wiped gently with a tissue paper around the tissue sections.
7. **Secondary antibody.** Addition100 µL of secondary antibody labeled to horseradish peroxidase (EnVision FLEX /HRP, SM802) to the tissue sections were they incubated in a humidity chamber at room temperature for 30 minutes. Sections were rinsed and immersed for 5 min in tow changes of buffer bath (E.V. F. Wash Buffer .SM831) removed. The excess buffer on the tissue sections by tapping and wiped gently with a tissue paper around the tissue sections.
8. **Dab apply.** 100 µL of freshly prepared DAB+ substrate-chromogen solution (prepared by apply one drop of E. F. DAB with Chromogen to 1 ml of E. F. Substrate Buffer), addition to the tissue sections, were incubated in a humidity chamber for 10 minutes. Sections were rinsed and immersed for 5 min in tow changes of buffer bath (E.V. F. Wash Buffer .SM831) removed. The excess buffer on the tissue sections by tapping and wiped gently with a tissue paper around the tissue sections.
9. **Hematoxylin Staining.** Sections were stained with Mayer's hematoxylin (Bio-Optica, Italy) for 3 min after which the stained sections were rinsed with tap water.
10. **Dehydration.** Dehydrate the tissue sections in three changes of ethanol alcohol for 2 minutes for each concentration of 70%, 90%, and 100%, respectively. Immersed Tissue sections in two changes of xylene for 10 min each, and mounted by (DPX) as mounting media, and covered with cover slips. Then examined the sections under a light microscope.

Ethical Approval:

Approvals were obtained from the Scientific Research Ethics Committees at the Al-Qadisiya University/ College of Nursing and College Veterinary Medicine, Iraq.

RESULTS

A total of 100 placenta samples were collected randomly from aborted women in the first months of pregnancy. After applying the Impression method, as is clear in **Figure 1**, and the examination, it became clear that the number of infected samples is 40 and constituted 40%, and the number of uninfected samples is 60, as is clear in **Table 1**.

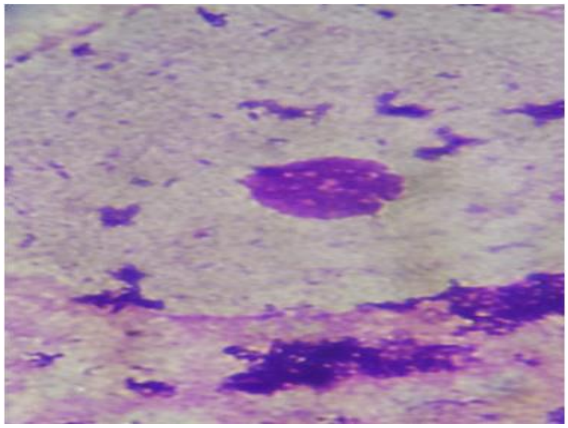

Figure (1): Cyst of Toxoplasma in placenta (Gimza Dye, 40X) by Impression method.

Table (1): It shows the number of positive and negative samples by Impression method

Method	Total specimens	The positive specimens	The negative specimen
Impression	100	40	60
Total percentage		40%	60%

PCR- RFLP

The GRA6 gene was amplified using the PCR method for all 40 positive tissue specimens, and 773 bp bands were detected under UV light after transferring the PCR products onto an agarose gel. In addition, MseI exonuclease (PCR-RFLP) was added to all positive specimens. After performing PCR-RFLP bands were detected 544 bp and 168 bp Type I, 623 bp and 75 bp Type II, and 544 bp and 97 bp Type III, and as it is clear in **Figure 2**. the number of the first, second and third types, the number of uninfected samples, and the specimens carrying DNA is not known, as shown in **Table 2**.

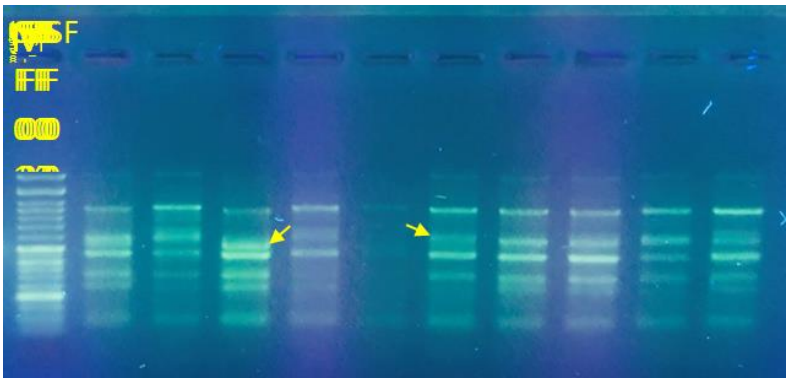

Figure (2): PCR-RFLP products of genotyping of *Toxoplasma gondii* based on an amplification of partial region of gene *GRA6* of *Toxoplasma gondii.*

Table (2): Samples for type I, type II and type III, the number of unknown samples and the number of uninfected samples.

Sample	Genotype I	Genotype II	Genotype III	Unknown	Negative
40	19	13	2	4	2
Percentage	47.5%	32.5%	5%	10%	5%

Immunohistochemistry method

A total of 38 of the 40 infected samples of the first method that were evaluated were positive for *T. gondii* by IHC and showed anti-gondii immune activity, as shown in **Figure 3**. Small circular cysts and pseudocysts containing bradyzoites were observed, and the infection rate was 95% of the samples, and only 2 of the placenta samples showed the absence of infection, as shown in **Table 3.**

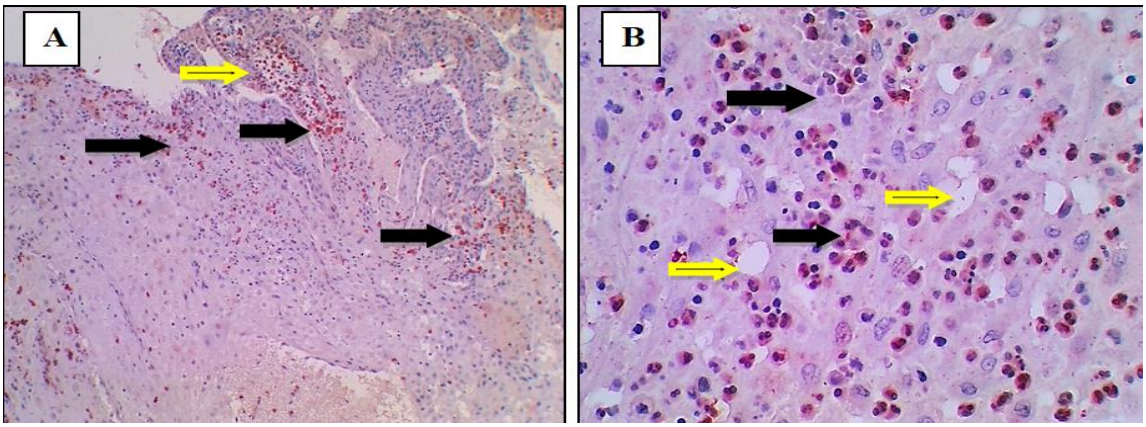

Figure (3): A&B/ Positive expression of anti-Toxoplasma gondii primary antibody (black arrow) indicated presence *Toxoplasma gondii* within epithelial cells of placenta with presence of spaces (yellow arrow) of necrotic epithelial in affected area.(Hematoxylin and DAB). A: 100x and B: 400x.

Table (3): Number of positive and negative samples by immunohistochemistry method.

Method	Total specimens	The positive specimens	The negative specimens
Immuno-histochemistry	40	38	2
Total percentage		95%	5%

DISCUSSION

The most important current methods used in research and studies to confirm toxoplasmosis are direct detection of parasites in tissues or body fluids, inoculation and isolation of protozoa in mice or in tissue culture and antibodies to IgM, IgG and IgA in serum or fluids [19].

Serological methods may be highly uncertain; for example, patients with immunosuppressive therapy or with an underlying disease causing a suppressed antibody response, and some individual differences for those with congenital toxoplasmosis [20]. The infection remains for months or years. Placental immunehistochemical testing in such cases can be very useful in terms of identifying the infective agent as well as in demonstrating a market increase in cytokines primarily produced by the original cells of the villi and embryonic membrane [21]. From the results of the current study, the three diagnostic methods (Impression, RFLP- PCR, and IHC) were specific, but IHC was more sensitive (100%) in documenting *T. gondii* infection in aborted women.

The current study aims, by means of an impression smear, to diagnose bradyzoite toxoplasmosis in the placenta from women who attended to maternal and child hospitals in Al-Diwaniyah Governorate. The total number of placenta samples (100 samples for each sample) was examined by impression smear, and the result of this study was 40 positive placenta samples. The parasite is transmitted from the uterus to the placenta and fetus. A different study of the parasite was recorded in several diagnostic methods or the result of placental and fetal tissues agreed with [22] who showed 78% positive in the placenta in Salah Al-Din Governorate and 70% in Al-Anbar Governorate, for placental tissue disorder with [23] who detected *T. gondii* parasite in 6 placentas of 24 women with 25%.

Toxoplasma isolated from humans was classified into three genotypes, type 1, type 2, and type 3. [24]. In general, the study of genotyping is necessary to understand the evolution of the population and the structure of parasites, especially after evolution in the molecular study and may help in identifying the sources of infection or the means of transmission to humans [25]. In most studies in North America and Europe, Toxoplasma type II was recorded as the most common toxoplasmosis infection in humans. [26] reported that the second genotype is the most common between 70 and 81% .

In this study, PCR-RFLP was applied to the forty samples, to show that the most common of genetic types was type I with 47.5%, type II rate 32.5%, type III rate 5% and the unknown was 10%. [27] A study in humans suggested that highly virulent and most prevalent strains of type I cause immunocompromised patients, while type II and type III are relatively harmless. Some studies have shown that *T. gondii* strains are of the first clonal type with acute virulence [28]. While [29] in Egypt, the study recorded a percentage of (44.4%). Of the positive cases were from Type I and 33.3% of the second type and two cases with 22.3% rate of unknown genotypes. In this study, the third type was not mentioned due to its absence in the samples examined.

The current study found a percentage of infection with Toxoplasma positive samples reached 95% by IHC method in the tested placenta. On the other hand, no cysts or tachyzoites of *T. gondii* were detected in goat tissues evaluated by IHC. The variability in these results may be

related to the different source of samples between humans and animals and the random distribution of the parasite may be a factor [30].

CONCLUSION

Several techniques can be used, but the immunohistochemistry technique is considered as a confirmatory test to classify aborted women into *T. gondii* infected women and non *T. gondii* infected women, and that PCR -RFLB method enables the identification of the most important species of *Toxoplasma* widespread in Diwaniyah, after confirmation from comparisons of diagnostic methods in the current study.

Ethical Approval: This was obtained from the Scientific Research Ethics Committees at the Al-Qadisiya University/ College of Nursing and College Veterinary Medicine, Iraq.

Conflict of interest: The authors declare no conflict of interest.

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Author contribution: Authors contributed equally in the study.

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