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Incidence Study For *Toxoplasma gondii* Depending On Molecular And Immunological Characterization In Recurrent Aborted Women

A Thesis

Submitted to the Council of the College of Medicine / University of Al-Qadisiyah in partial Fulfillment of the Requirements for the master Degree of Science in

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By

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بِسمِ اللهِ الرَحمِن الرَحِيم

((قَالُوا سُبْحَانَكَ لا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ))

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Date : / / /2018

Report of the Head of Department of Microbiology

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Dedication

This Work is Dedicated

To

Soul my mother , my father and

my love … my wife

For her patience, tender care and

constant support.

Falah

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A case- control study was conducted in Al-Najaf province . Five ml blood samples obtained from seventy recurrent aborted women suspected with toxoplasmosis attending to AL-Zahraa maternity and children teaching hospital for the period from December 2017 to March 2018, and compared to (20) healthy women as a control group, their ages ranged between 16-42 years, three ml blood samples were assessed for serum detection positivity of latex agglutination test , Enzyme Linked immune sorbent assay (immunoglobulin G and immunoglobulin M).Two ml blood sample was utilized for genomic DNA extraction from peripheral blood and (2-5) gram of fetoplacental tissue for detection of *B1* gene using nested polymerase chain reaction technique. The distribution analysis showed that the highest toxoplasmosis among recurrent aborted women.

The results showed a high positive of toxoplasmosis in the latex agglutination test and Enzyme Linked immune sorbent assay were 44(62.9%) and 33(47.1%) out of (70)respectively. The results were confirmed using nested polymerase chain reaction technique. The positive for *B*1 gene was 12(17.1%) and 24(34.3%) out of (70) of the blood sample and the tissue sample respectively. No positive cases of anti-*Toxoplasma gondii* IgM antibodies were detected.

In addition, the current study confirmed that nested polymerase chain reaction tissue was more sensitive (100%) and specificity (66%) than other techniques used and at a high level of significance where (*P* value= 0.02).

This study concluded that nested polymerase chain reaction technique was one of the best techniques for diagnosing of toxoplasmosis infection through the investigation of *B*1 gene in tissue sample.

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List of Abbreviation

Abbreviation	Meaning
µl	Microlitter
AIDS	acquired immune deficiency syndrome
bp	base pair
CD	Cluster of differentiation
CFT	Complement fixation test
CSF	Cerebral spinal fluid
DAT	Direct agglutination test
df	degree of freedom
DNA	Deoxyribonucleic acid
DS	Double stranded
DT	Dye test
EDTA	Ethilin diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
HRP	Horseradish peroxidase
IG	Immunoglobulin
IHAT	Indirect hemagglutination test
LAT	Latex agglutination test
nPCR	Nested Polymerase Chain Reaction
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PH	Power of hydrogen
RFLP	restriction fragmented length polymor- phism
RT-PCR	Real-time Polymerase Chain Reaction
SFDT	Sabin Feldman Dye Test
TBE	Tris borate EDTA
TBE	Tris-Boric acid - EDTA
TH	T helper
TMP	tetramethylbenzidine
FATG	Favor tissue genomic
FABG	Favor blood genomic
U.V.	Ultra violate
IFAT	Indirect immunofluorescence antibody test

Chapter One
Introduction & Literatures Review

Introduction and Literature Review

1.1. Introduction

Toxoplasmosis is one of the most common parasitic diseases where approximately one-third of the world's population is affected (Robert-Gangneux *et al*., 2017). Its capable of causing severe and life threatening conditions specially in pregnant women and immunecompromised individuals (Opsteegh *et al*. 2016).

Human infections generally occur by the consumption of undercooked meat that contains tissue cysts or by water and food contaminated with oocysts present in cat feces (Jafari *et al*., 2012).The infection is transmitted directly from the mother to the fetus during the transition of the active phase tachyzoite across the placenta, rarely through blood transfusions or organs transplantation

(Vimercati *et al*., 2017).

Congenital infection is one of the most important sequels of toxoplasmosis in pregnant women (Mahmood, 2016). Congenital transmission of *Toxoplasma gondii* predominantly occurs at the first time during pregnancy (Paquet *et al*., 2013). The severity of congenital toxoplasmosis is highest in the first and second trimesters of pregnancy which usually results in abortion or stillbirth **(**Ghasemi, 2016).

Several serological diagnostic methods were used for *T. gondii* identification like Latex agglutination Test, enzyme linked immune sorbent assay (Pittman and Knoll , 2015).

Latex Agglutination Test (LAT) is easy to perform and commercially available in kit form, LAT tests do not require species-specific anti-sera or conjugates and since it is available in kit form they have become popular for

sero-diagnosis of toxoplasmosis, however, antibodies detected by LAT may appear later in infection than those detected by tests such as Dye test (DT) and IFAT and their sensitivity may therefore be low especially in acute infections ,the Enzyme Linked Immune sorbent Assay (ELISA) for *Toxoplasma gondii* antibodies has been adapted for use in human and most domestic animals and modified methods have been developed for the detection of *Toxoplasma* antigen in body fluid, because of its ease of use, cost effectiveness and high sensitivity and specificity, it has replaced older tests in many laboratories (Mohamed *et al*, 2012).

Recently, the polymerase chain reaction (PCR) technique has been widely used due to its highly sensitivity and specificity of *T. gondii* detection (Sultan & AL-Fatlawi, 2016). polymerase chain reaction is the only method that can detect *T. gondii* organisms in low numbers (10 organisms per ml) and can detect a partly destroyed parasite (Turkey *et al.*, 2014). The most often used target for PCR detection is the 35 fold repetitive *B1* gene (Burg *et al.*, 1989).

1.2. Aim of study

The present study has been conducted to detected of toxoplasmosis in recurrent aborted women by serological and molecular technique .

This aim was achieved by the following objectives:

1-Identification the incidence of *T .gondii* infection in recurrent aborted women.

2- Determine the most accurate test that can be used in diagnosis this parasite (LAT, ELIZA [IgG & IgM], nPCR for Blood and Tissue) .

3- Conduct the validity of nested polymerase chain reaction (nPCR) by used *B*1 gene from blood and fetoplacental tissue.

1.3. Literatures Review

1.3.1. History of *Toxoplasma gondii*

Toxoplasma gondii was firstly discovered in laboratory animals by Charles Nicolle & Manceaux in 1908,they described this parasite as Leishmania like in the gondis (African rodent) in Pasture institute in Tunisia, In the same year, Alfonso Splendore described the parasite at the Portuguese hospital in Sao Paulo, Brazil, the name *Toxoplasma gondii* was given to the parasite by Nicolle & Manceaux in 1908 after they agree that the parasite was different from Leishmania & Piproplasma (Veronesi *et al.*, 2017).

The name of *Toxoplasma gondii* was derived from a Greek word toxon, which mean arc, referred to lunate shape organism in fresh state, the specific name of the gondii was drawn from rodent *Ctenodactylus gondii* (Ching, 2016).

Toxoplasma gondii an obligate intracellular protozoan parasite, is a significant pathogen in humans, the parasite whose final host is the felidea is scattered in nature and infects numerous other mammals (Silva, 2012).

It was observed by Hutchison in 1965 and confirmed by others when cats were fed *Toxoplasma* via infected mice, the infection could be transmitted back to mice through cat's faces even after storage in water up to a year or more, transmission from cat to mouse was accomplished by sporocysts pass in cat faces (Waap *et al*., 2016).

1.3.2. Taxonomy

The final classification of *Toxoplasma gondii* mentioned by Roberts and Janovy (2000) .

Kingdom	Protesta
Phylum	Apicomplexa
Class	Sporozoa
Subclass	Coccidiasina
Order	Eucoccidiorida
Suborder	Eimeriorina
Family	Sarcocystidae
Subfamily	Toxoplasmatinae
Genus	*Toxoplasma*
Species	*gondii*

1.3.3. Epidemiology

Many factors including management , food processing , density of cats or wild felines and environmental conditions (that have an effect on Oocysts sporulation) play an important role in the epidemiology of *Toxoplasma gondii* (Tenter *et al*., 2000) .

Niazi *et al.* (1988) said that, 60% of Iraqi women of childbearing age were susceptible and might acquired the infection during pregnancy.

In Iraq; Kader& Al-Khayat (2013); Al-Hamdani and Mahdi (1996) in Mosul and Basra respectively reported that ,the infection rate in recurrent aborted women were 40.25% and 18.5% respectively .

AL-Timimi (2004) observed, by using latex and IFAT methods, among 168 women with a history of abortion in Baghdad the rates were 44% and 29% respectively. In Mosul, AL-Ubaydi (2004) compared the seropositivity by applying three different laboratory methods on a total of 406 sera; latex test

showed 79 % followed by active infection 46 % using Dye test (DT), while using ELISA technique gave37%.

Al-Ramahi *et al.* (2005) reported that, the rate of *Toxoplasma* infection in Al-Diwanyia province was 49.65% with market variation according to professional categories as 56%, 28.27%, 44.82%, 60.86% 68.5% in veterinarians, veterinary students, urban women, rural women, and butchers respectively.

Al-Rawi (2009) found that the rate of infection was 4.16%, 25.83%, 15.83%for aborted women in Baghdad using ELISA (IgM, IgG) and nPCR techniques respectively.

Al-Shikhly (2010) recorded that the rate of toxoplasmosis in Baghdad among healthy females was 51.52%, 41%, 16.2% by using agglutination test (LAT), ELISA IgM and IgG techniques respectively.

In Kut province Al-Mayahi (2011) found that the rate of *Toxoplasma* infection was 66.35 %, 44.71% in women using ELISA IgM and IgG technique respectively.

Hasson, (2004) recorded that, the rate of *Toxoplasma* infection among the pregnant women in Al-Najaf province was 40.4%.

in Egypt Ahmed *et al*. (1996) reported that, *Toxoplasma* infection rate was 26% by using IFAT. Another study by AI – Qurashi, (2004) in eastern region of Saudi Arabia, a high *T. gondii* percentage 52.1% was reported among donors in Asir.

In Jordan, Jumaian *et al*. (2005) found that, the rate of toxoplasmosis was 31,6% among pregnant women.

In Qatar Abu-Madi and Haydee (2010) showed that among 823 woman of child bearring age, 35.1% and 5.2% tested positive for *T . gondii* IgG and IgM respectively.

In Turkey, sero survey of Toxoplasmosis among women denoted that 49.4% are positive (Altintas *et al*.,1997).

In Brazil toxoplasmosis is an important public health problem with serological prevalence ranging from 50-54% in adults (Bahia-Oliveira, 2003). In United States between 3% and 67% (Wu and Garcia, 2005).

Zamani *et al*.(2007) reported that *T.gondii* infection has shown considerable prevalence in Iran and seropositivity ranges from 5.7-78%.

1.3.4. Morphology and Structure

The parasite is a coccidian which consists of three forms (Hill & Dubey 2018)

1- Oocyst (Resistant form in the external environment).
2- Tachyzoites (Rapid multiplication stage).
3- Bradyzoites(Slow multiplication stage).

1.3.4.1. Oocyst

The oocyst is spherical or ovoidal, protected by very resistant membrane consist of two layers and measure 10-12 micrometer (µm) in diameter (Gane, 2001). It's formation occurs in the intestine of the definitive host (cat) by gametogenesis (sexual reproduction) and then excrete in cat faces to the external environment as unsporulated oocyst (Dubey, 2016).

Dubey (2003) reported that, after 1-5 days depending on surrounding condition the sporulation will occur and the diameter of sporulating oocyst will be measured (11-13 µm).The sporulated oocyst is characterized by containing two sporocysts, each one contain four sporozoites (8×2 µm) (Dubey, 2016).

1.3.4.2.Tachyzoite

Tachyzoite is the actively proliferating trophozoite which is observed during the acute stage of infection ,the intracellular infection can occur in all mammalian cells except reticulocyte (Ferguson & Dubremetz, 2014).

Montoya and Lisenfeld (2004) described the tachyzoites as a crescent in shape, with one end pointed and the other rounded, it measures approximately 2-4 µm width & 4-8 µm length, the nucleus is ovoid and situated near the blunt end of the parasite, when the trophozoites are stained with Giemsa stain, the

cytoplasm appearing azure blue and the nucleus red ,*Toxoplasma gondii* tachyzoites survive in 0.5% trypsin solution for one hour and occasionally survive for two hours in acid pepsin solution (Dubey *et al*., 1998).

1.3.4.3.Bradyzoite

Bradyzoites is slowly multiplying organisms contained in pseudocyst(tissue cyst), the later can exist in any organ, but are commonly found in myocardium, skeletal muscles & the brain ,The encysted parasites can persist throughout the life of the host (Jeffers *et al.*, 2018).

The tissue cyst is rounded or oval, 10-200 µm in size and contains numerous bradyzoites, it remains viable at 52 C˚ for 5-9 minutes and is rendered non-viable by heating to 61 C˚ or higher temperature for 3-6 minutes (Dubey *et al*., 1990).

1.3.5. Life cycle

Toxoplasma gondii in general have complicated life cycles, It is capable of infecting and replicating within virtually any nucleated mammalian or avian cell (Dubey and Lindsay, 2004). The life cycle is divided between feline and non-feline infections (Figure 1), which are correlated with sexual and asexual replication respectively (Black and Boothroyd, 2000).

1.3.5.1. Sexual (Enteroepithelial) cycle

The sexual cycle of *T. gondii* occur in cats in which they are the definitive hosts of *T. gondii* ,this cycle is initiated when a member of the cat family (Felidae) ingests either Oocysts or tissue cysts, the wall of the tissue cyst is digested by the proteolytic enzymes in the stomach and small intestine releasing bradyzoites which penetrate epithelial cells of the small intestine and initiate development of numerous generations of asexual schizonts (Dubey, 2004).

The organisms (merozoites) released from schizonts form male and female gametes (gametogony), after the female gamete is fertilized by the male gamete, oocyst wall formation begins around the fertilized gamete, oocysts of *T. gondii*

are formed only in cats (Smith & Rebuck,2000).The definitive hosts of *T. gondii* excrete oocysts (3–10 days) after ingesting tissue cysts/bradyzoites (18 days) and after ingesting oocysts, and (13 days) after ingesting tachyzoites (Dubey, 2002).

1.3.5.2.Asexual (Extraintestinal) cycle

Asexual cycle occurs in intermediate hosts, this cycle is initiated after ingestion of sporulated oocysts or tissue cyst, sporulated oocysts are infectious to humans and to all other warm-blooded hosts, upon ingestion of sporulated oocysts, sporozoites are liberated in the gut lumen(Dubey, 2004) .Sporozoites enter intestinal epithelial cells, become tachyzoites and multiply in the intestine and associated lymph nodes before circulation to other organs via lymph and blood,the tachyzoite multiplies asexually within the host cell by repeated divisions,after a few divisions *T. gondii* form another stage called (tissue cysts). Tissue cysts grow and remain intracellular (Singh, 2003).

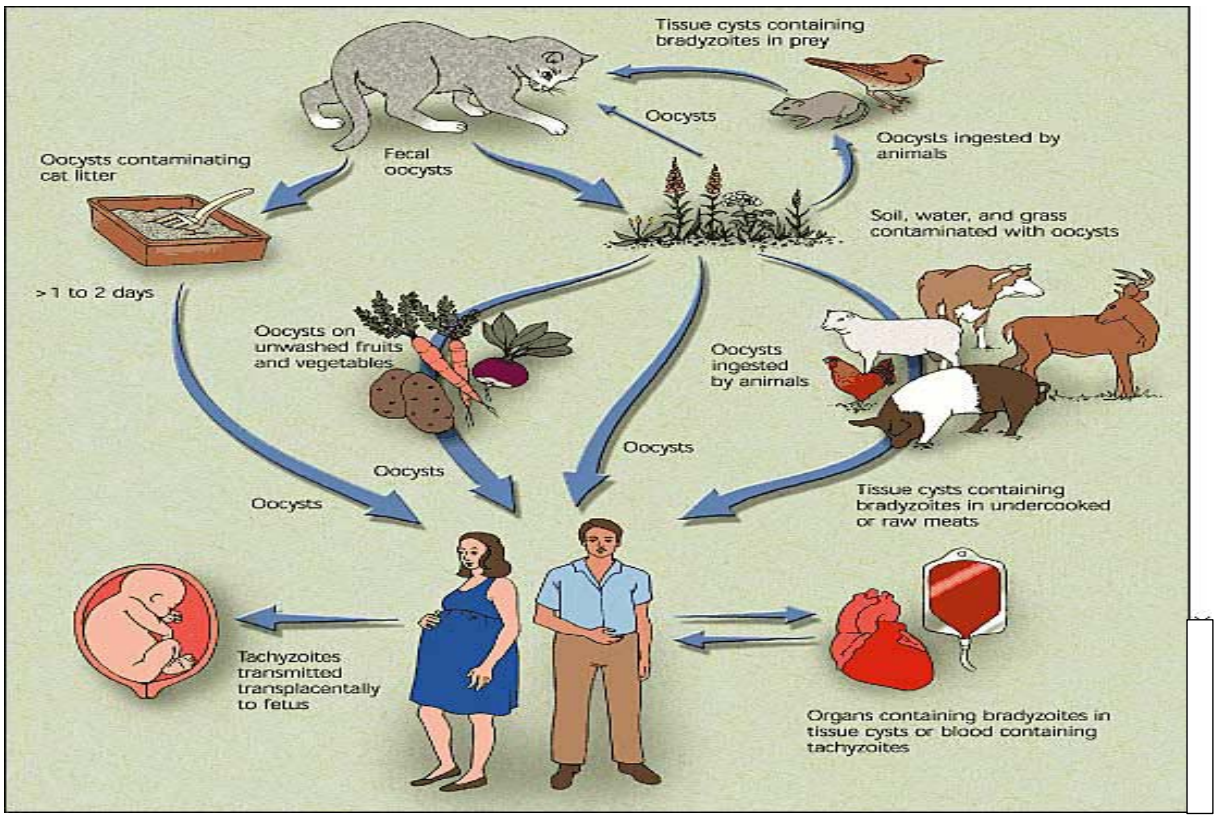


Figure (1): Life cycle of *Toxoplasma gondii* (Al-Saadii, 2013) .

Although tissue cysts may develop in visceral organs, including lungs, liver, and kidneys, they are more prevalent in muscular and neural tissues, including the brain, eye, skeletal, and cardiac muscle (Kelley *et al*., 2005; Dubey, 2007).

1.3.6.Transmission Routes

There are five recognized modes of transmission to humans (Bhopale, 2003).

1-Ingestion of the oocyst via unwashed vegetables.

2-Consumption of undercooked meat that containing the cyst.

3- Blood transfusion or human organ transplant, a risk associated with bone marrow which is possible if the donor is parasitemic at the time of collection.

4- Accidental inoculation e.g. contaminated needles from work with infected animals.

1.3.7. Congenital toxoplasmosis

The fetus can gain the infection after colonization of tachyzoite in the placenta of pregnant women who acquired the primary infection (Thiebaut *et al*., 2007).The frequency of vertical transmission increases with the gestational stage at maternal infection (Williams *et al*., 2005).

The transplacental passage of tachyzoites is rare during the first period of pregnancy, Although the consequences for the offspring are heavy (Pfaff *et al.*, 2007).

Women infected with *T. gondii* before conception do not transmit the infection to their fetuses, but there is some exception, women infected with *T. gondii* during pregnancy can transmit the infection across the placenta to their fetuses (Remington *et al*., 2001). However, if infection is acquired in the first trimester is the more severity of disease (Remington *et al.*, 2006).

1.3.8. Strains of *Toxoplasma gondii*

Toxoplasma gondii is globally distributed protozoan parasites that can infect virtually all warm blooded animals and humans, despite the existence of sexual phases in the life cycle (Kopeena *et al*., 2006). *T.gondii* has unusual structure

dominated by three clonal lineages that predominate in North America & Europe, molecular genotyping has shown that approximately 90% of *T.gondii* isolates that have been analyzed can be classified into three lineage Type I, II, III (Zhou *et al*., 2009).

Virulence in laboratory mice ,isoenzyme pattern analysis and restriction fragmented length polymorphism (RFLP) have been used to differentiate *T. gondii* strains into those three distinct clonal lineages (Vallochi *et al*., 2005).

Type I and type II strains have been associated with human congenital toxoplasmosis (Fox and Bzik, 2002).Type I is highly virulent in murine infections, whereas type II is relatively avirulent and type III is of intermediate virulence in mice (Peyron *et al*., 2006).

The marked virulence of type I strains of *T. gondii* is typified by the commonly used laboratory strain RH, was isolated from a child with lethal case of toxoplasma encephalitis, Parasites strain type may also play a role in determining the severity of the human infection (Sibley, 2003).

Most cases in humans were caused by type Π strains which are significantly more often associated with reactivation of chronic infections and accounted for 65% of strains isolated from acquired immune deficiency syndrome (AIDS) patients (Taylor *et al*., 2006).

1.3.9. Risk factors for Toxoplasmosis

Toxoplasmosis is important due to the possibility of transplacental transmission and harming the fetus (Boyer *et al.*,2005). The rate of risk for pregnancy depends on infection prevalence among women at childbearing age, the degree of contact between pregnant women and protozoan transmission sources, and the stage of gestation when transmission occurs (Mariza *et al.*, 2004).

Immunological state of the individuals and favorable environmental conditions such as hot weather, for the survival of the oocysts, which are

discarded in cat feces and genetic factors play very important role in incidence of toxoplasmosis (Carter, 2013).

Women who get infection during pregnancy may show a variety of clinical signs and symptoms depending on many factors, such as the number of parasites, virulence of strain, genetic background and the time period the mother acquires infection (Montoya & Liesenfeld, 2004). If the mother is infected in the first trimester, the result is abortion, stillbirth or severe disease of fetus (Tenter *et al*., 2000).

1.3.10. Clinical sings of toxoplasmosis

Toxoplasma infection is classified into acute and chronic stages, Acute or early stage is mostly associated with the proliferative form (tachyzoite) while the tissue cyst is the predominant form during chronic infection (Waree, 2008).

During acute infection the proliferation of the tachyzoite in host tissue leads to host cell death, rapid invasion of neighboring cells and disseminate via the bloodstream, and can invade many tissues, such as liver, spleen and brain (Waree, 2008).

Most immunocompetent people infected with *T. gondii* are asymptomatic (Dubey and Jones, 2008). Lymphadenitis is the most common clinical form of toxoplasmosis and may be accompanied by a number of nonspecific symptoms such headache, fever, malaise, fatigue, sore throat, and myalgia, Lymphadenopathy caused by toxoplasmosis usually occurs in the neck region (Hill *et al*., 2005).

Toxoplasma gondii only becomes a threat to humans when the host immune system is unable to control any ruptured cyst, fetuses that don’t have fully developed immune system and individuals with weakened immune systems are unable to protect themselves from *T. gondii* infection, In immunocompromised patients, toxoplasmosis mostly happens as a result of reactivation of chronic infection, In these patients, clinical symptoms consist of mental status changes,

seizures, sensory abnormalities, movement disorders, and neuropsychiatric findings (Dalimi & Abdoli 2012).

The acute or reactivated toxoplasmosis during pregnancy leads to transplacental transmition of organisms to fetus As, tachyzoites by pass the placental blood barrier and invade the fetal organs to propagate and compromise the embryonic developmental process (Dubey *et al*., 2012). The primary infection of pregnant women with *T. gondii* may cause spontaneous abortion and stillbirth (Pappas *et al*.,2009).

Classic signs of congenital toxoplasmosis in live-born infants include retinochorditis, hydrocephalus or microcephalus and cerebral calcification (Andrew & Nandini, 2012). Ocular disease is the most common manifestation of congenital toxoplasmosis and retinochoroiditis may occur later in life in an otherwise healthy child who was exposed to *T. gondii* in utero (Butler *et al*., 2013).

Congenital Toxoplasmosis is most severe when the mother becomes infected in the first trimester, approximately 10 to 20% of fetuses are infected, If the infection is acquired in the second trimester 30 to 40% of fetuses are infected, but the disease is mild or asymptomatic at birth (Muzzio *et al*., 2014). These differences in transmission may be related to the placental blood flow, size of uterus, or to the immunocompetence of the mother (Singh, 2003).

The most common form of the disease is latent (asymptomatic), actually after ingestion of the parasite and proliferation of tachyzoites in various organs during the acute stage, the parasite forms cysts in the brain and establishes a chronic infection in human (Carruthers & Suzuki, 2007). So, the infection may cause various hormonal and mental disorders (Abdol *et al.*, 2011).

Toxoplasma gondii has been implicated in the pathogenesis of many diseases, most notably schizophrenia (Horacek *et al*., 2012) but also depression and suicide attempts (Zhang *et al*., 2012).

Tissue cysts are formed mainly in the CNS, muscles, and visceral organs, and probably persist for the life of the host (Hill& Dubey, 2018). The mechanism of this persistence is unknown ,However, many investigators believe that tissue cysts break down periodically, with bradyzoites transforming to tachyzoites that reinvade host cells and again transform to bradyzoites within new tissue cysts (Tandon *et al*., 2010).

Encephalitis is the most important manifestation of toxoplasmosis in immunosuppressed patients as it causes severe damage to brain and death (Shirbazou *et al.,* 2015).

Ocular toxoplasmosis is a condition that develops in some people after toxoplasmosis parasite infection (Lambert *et al*., 2006). The infection may be transmitted because of contamination, or it may be transmitted from an infected mother to a baby during pregnancy (Soheilian *et al*., 2011).

Lupi *et al*.(2009) showed that, eye infection leads to acute inflammation of the retina, which resolves leaving scarring, symptoms include eye pain, blurred vision, and photophobia. Eventual blindness will result if the central structures of the retina are infected by the parasite and if untreated (Jeffrey *et al*., 2001).

1.3.11. Symptoms and Complications of Toxoplasmosis

When a person becomes infected with *T. gondii* they may experience mild flu-like symptoms that last for several weeks and then go away, such as tender and muscle aches (Dalimi and Abdoli, 2012).

In immunocompromised patients, toxoplasmosis is life threatening whatever the strain of the parasite, contrasting with the setting of *Toxoplasma* infection in immunocompetent subjects, yet the host immune background is of great importance (Mai *et al.,* 2009).

Various factors responsible for impaired cellular immunity can lead to severe toxoplasmosis, among which are HIV infection and immunosuppressive therapies (Derouin and Pelloux, 2008). Patients are more commonly at risk for

disease reactivation resulting from cyst rupture than for a newly acquired infection, but the risk may differ among categories of patients (Martina *et al.*, 2011).

Many organs can be involved, either because they are target for encystment and thus are subsequent potential sites for cyst reactivation or because they are secondarily infected following the dissemination of parasites from an initial reactivation site, like brain, lungs and eyes (Jones *et al*., 2002). And heart, resulting in myocarditis, but the isolation of *Toxoplasma* from many other sites, such as liver, pancreas, bone marrow, bladder lymph nodes, kidney, spleen, and skin has been documented (Patrat-Delon *et al.*, 2010).

McLeod & Remington (2007) showed that signs and symptoms in immunocompromised patients include, headache, confusion, seizures, lung problems that may resemble tuberculosis or pneumocystis, blurred vision caused by severe inflammation of the retina (ocular toxoplasmosis).

Early infections of congenital toxoplasmosis may lead to stillbirth or miscarriage, and children who do survive are likely to be born with serious problems, such as Seizures, abnormal enlargement or smallness of the head, an enlarged liver and spleen, jaundice, severe eye infections, and mental disability (Sharenda et al., 2009).

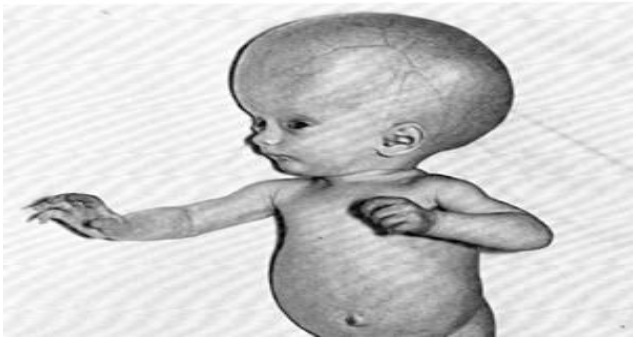


Figure (2):Congenital toxoplasmosis (hydrocephaly)(AL-Maamuri, 2014).

1.3.12. Immunity

The host defense mechanisms against infection with *T. gondii* consist of innate immunity, which mediate the initial protection and adaptive immunity, that is divided into humoral and cell- mediated immunity **(**Filisetti and Candolfi, 2004).

The major surface antigen in *Toxoplasma* was a family of related proteins and identified that this antigen coats the surface of the parasite and may be involved in the attachment and/or regulation of the host immune responses (Wasmuth et al., 2012). *T. gondii* tachyzoites are highly virulent and for the survival of the host it is an important that proliferation and spreading of parasites should be controlled (Frenkel, 1988).

Directly after ingestion of *T. gondii* the host innate immune response recognizes and responds to the protozoan as a foreign antigen (Denis and Ermanno, 2004).The first line of defense against *T. gondii* is the intestinal epithelia, after ingestion of tissue cysts or oocysts of *T. gondii* , bradyzoites or sporozoites are released in the small intestine and actively penetrate enterocytes, the infected enterocytes will secrete cytotoxic molecules such as nitric oxide and also chemokines, which chemoattract immune cells such as neutrophils, Dendritic cells, macrophages, monocytes and T cells (Mennechet *et al*., 2002).

T. gondii triggers the activation of macrophages in the tissues along with Natural killer cells, which begin an attempt to remove the invader, The objective of the innate immune response is not to entirely eradicate the invader, however, for maximum the proliferation of the parasite and to signal for the activation of the adaptive immune response (Yingst and Hoover, 2003). The innate immune response begins straight after infection and diminishes approximately two weeks after the initial infection by *T. gondii* (Denis and Ermanno, 2004).

Humoral immunity also called the antibody-mediated beta cellularis immune system, is the aspect of immunity that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins and certain antimicrobial peptides, humoral immunity is so named because it involves substances found in the humours, or body fluids (Pier *et al*.,2004).

Humoral immunity refers to antibody production, and the accessory processes that accompany it including: T helper2 activation and cytokine production, germinal center formation and isotype switching, affinity maturation and memory cell generation, It also refers to the effector functions of antibody, which include pathogen and toxin neutralization, classical complement activation, and opsonin promotion of phagocytosis and pathogen elimination (Janeway, 2001).

The adaptive immune response will commence and produce specific antibodies and effector cells to remove *T. gondii*, the innate immune response signals the differentiation of macrophages, B cells, and dendritic cells into antigen presenting cells, Antigen presenting cells may after that presents the antigen to T cells, exciting the differentiation of T cells into effector cells, counting CD8 cytotoxic T cells, to directly kill *T. gondii*, Specially in humans the infection of dendritic cells by *T. gondii* that leads to the activation of CD40, which induces the construction of effector cells, which can directly kill or secrete cytokines to recruit other cells to eradicate the pathogen (Denis and Ermanno, 2004).

A balance between innate and adaptive mechanisms leads to pro-inflammatory and regulatory responses in the immunopathology of toxoplasmosis (Lang, 2007).

Denis and Ermanno, (2004) referred that immunological memory will be developed in response to the toxoplasmosis infectivity, this immunological

memory can defend the host from reinfection It is hypothesized , so as to this immunological memory is responsible for the rupturing of intracellular cysts caused by *T. gondii* .

1.3.13. Diagnosis

Clinical signs of toxoplasmosis are nonspecific and not sufficiently characteristic, so diagnosis of *Toxoplasma gondii* infection in human is made by several important methods which are biological, serological, histological and molecular methods (Sinjin et *al*. 2004).

1.3.13.1.Direct Microscopical Examination

Demonstration of tachyzoites in fluid specimens such as blood, CSF, bronchoalveolar lavage, milk, eye secretions, saliva and urine or in smear which is made from a biopsy or autopsy of skeletal muscle, lung, brain and eye to reflect the presence of tissue cyst (Piekarski, 2012).

The tachyzoite are usually crescent, round or oval in shape and tissue cysts are usually spherical and lack septa (Aaiz, 2010).

The biopsies may demonstrate tachyzoites or cysts, that stains with hematoxylin and eosin in routine histopathological preparations,The Romanovsky stains, for example Geimsa and Wright's, also demonstrate *T. gondii* forms well (Remington & Thulliez, 2004).

1.3.13.2. Serological Tests

Several serological events are available for the detection of *T. gondii* antibody in patients, which may assist diagnosis, these include:

1.3.13.2.1. Latex Agglutination Test (LAT)

It is a modified agglutination test in which antigen coated latex particles have been used as a carrier of the antigen ,This test is used normally for the diagnosis of Toxoplasma IgM and IgG Abs, Titers rise rapidly during the acute phase, the reaction allows visual observation of the antigen-antibody complex (Ye *et al.*, 2011). It is simple to perform, cheap and sensitive to be used as a screening test (Holliman, 2003).

1.3.13.2.2. Enzyme Linked Immuno sorbent Assay (ELISA)

The enzyme-linked immune sorbent assay (ELISA) represents an important technique used mainly to interested detects of IgG and IgM through series steps that made on the patient's serum (Iddawela *et al*., 2015).

Kamal *et al*. (2014) referred that ELISA technique helpful in diagnosis infected women within the period 4 to 5 months and that are very important to detection *T. gondii* in pregnant women in their first months of gestation.

Capobiango *et al*. (2015) found that ELISA the most method used for diagnosis, which is the widest spread of roads that can identify the different stages of infection, especially in aborted women with toxoplasmosis. Frischknecht *et al*. (2011) recorded that ELISA gold assay for diagnosis of toxoplasmosis in pregnant women and for differentiation between acute and chronic infections.

Detection of toxoplasmosis directly depended on the positive or negative of serological methods that looking for anti-*T. gondii* specific antibodies in the patient's serum sample (Tekkesin *et al*., 2011). In Iranian study of Ahmad *et al*. (2014) showed that, serological methods consider more efficient diagnostic tests to detect *T.gondii* in women.

Fatimah *et al*. (2015) reported that serology survey findings relationship between toxoplasmosis and abortion or stillbirth quotient in women.

1.3.13.3 Polymerase Chain Reaction (PCR)

PCR is a molecular biology and biochemistry technique used to amplify many copies of a region of DNA that lies between two regions of Known sequence (Smithsonian, 2006).

Schönian *et al*. (2011) reported that PCR is commonly used in medical and biological research for a diversity of tasks, such as the recognition of hereditary diseases, diagnosis of infectious diseases and identification of genetic fingerprints.

Su *et al*. (2010) said that the purpose of a PCR is to make a huge number of copies of a gene this is necessary to have enough starting template for sequencing.

Al-kalaby (2008) mentioned that, PCR amplification of parasite DNA from tissue, CSF, amniotic fluid or blood is a sensitive method for detection of infection and result of PCR do not depend on the immunological status of the patient.

There are many PCR principles used for detection of *T.gondii* DNA and they are as follows:

1.3.13.3.1. Conventional PCR

The PCR is based on the same principles for copying DNA as those found in nature, the building blocks of DNA are four nucleotides, represented by the letters A (adenine), C (cytosine), G (guanine), and T (thymine),(Campos and Quesada, 2017).

PCR is performed by denaturation of double stranded DNA to generate single stranded DNA molecules, Primers ,which are short single stranded oligonucleotides, anneal to the single stranded DNA molecules at specific locations, the design of the primers determines which DNA sequence that will be amplified, Next a polymerase enzyme recognizes annealed primers and initiates elongation. Double stranded (ds) DNA molecules are synthesized (Paredes *and Villar –Rodil* 2016) .

Repeat the process in the number of courses and quantity increase number of copies the DNA in each cycle, the large amount of copies of a specific DNA pieces are normally analyzed using a gel electrophoresis processing, and can be visualized by staining with ethidium bromide and illumination with ultra violet light (Chimbevo, 2013).

1.3.13.3.2. Nested PCR

Nested PCR is suitable for detection of low copy numbers of target DNA against a high background of host tissue DNA and inhibitors of DNA polymerase *T. gondii* can be detected by this technique ,*T.gondii* DNA can be isolated and detected the use of nested PCR (Jalal *et al*. , 2004).

This approach is more successful than diluting and reamplifying with same primers. *T.gondii* can be detected by this technique (Jalal *et al*., 2009;Monttoya *et al*., 2009). Moreover different strains of *T.gondii* can be identified by using this type of PCR (Zakimi *et al*., 2006).

Wahab *et al*. (2010) found that, the *B1* gene, which is present in all *T. gondii* strains, has been the most frequently used target in PCR. A variety of specific gene targets has been described such as multicopy gene sequences as the 35-fold-repeated *B1* gene (Burg *et al.,* 1989). Described 529-bp repetitive sequence (AF146527 sequence), which occurs in 200 to 300 copies per genome, seems to be a promising target for sensitive diagnostic PCR assays (Homan *et al*., 2000).

1.3.13.3.3. Real time PCR

Real-time PCR was developed for the diagnosis of toxoplasmosis. Real-time PCR uses a fluorescence-labeled oligonucleotide probe, which eliminates the need for post-PCR processing (Loeffler *et al*., 2002). As well as, RT-PCR for DNA in amniotic fluid with 98% sensitivity and 100% specificity of toxoplasmosis (Teixeira *et al*., 2013).

The use of real-time PCR and automated methods for DNA extraction will result in a decrease in the inter laboratory variability observed with the conventional three-stage PCR (Yera *et al*., 2009). In addition real-time PCR can be used to estimate the concentration of parasites in amniotic fluid, which may be helpful for physicians to assess neonatal outcome (maternal infections acquired before 20 weeks with a parasite load of >100/ml of amniotic fluid were found to have the highest risk of severe fetal outcome), (Paquet, 2013).

1.3.14. Control and prevention

To prevent infection of human beings by *T. gondii*, people handling meat should wash their hands thoroughly with soap and water before going to other tasks, all cutting boards, sink tops, knives, and other materials contacting uncooked meat should also be washed with soap and water, tasting meat while cooking or while seasoning should be avoided, washing is effective because the stages of *T. gondii* in meat are killed by contact with soap and water *T. gondii* in meat are killed by exposure to extreme cold or heat (Hill, 2005). Tissue cysts in meat are killed by heating to an internal temperature of 67 ºC Freezing meat overnight in a household freezer at -13 ºC is effective in killing most tissue cysts, Salting and the addition of products to meat to enhance color and taste can have deleterious effects on the viability of *T. gondii* in meat *T. gondii* in tissue cysts or oocysts is killed by exposure to 0.5 krad of gamma irradiation (Dubey, 2010).

Pregnant women should avoid contact with cats, cat litter, soil, and raw meat Pet cats should be fed only dry, canned, or cooked food and the cat litter box should be emptied daily to prevent sporulation of oocysts, Gloves should be worn while gardening and vegetables should be washed thoroughly before eating because they may have been contaminated with cat feces, People should avoid drinking unfiltered water from lakes, ponds (Sakikawa *et al.*, 2012).

Chapter two
Materials and methods

2. Materials and methods

2.1. Materials

2.1.1. Laboratory Instruments and equipment's.

Table (2-1) the equipment's and instruments that used in this study with their manufacturer and Origin.

Equipments and instruments	Manufacturer and Origin
Centrifuge	kokusan /Japan
Deep freezer -80°C	Jermaks/Germany
Digital camera	Samsung /China
Disposable syringe 5 ml	Sterile EO. / China
Electric oven	Memmert /Germany
Electrophoresis	Biometra /USA
ELISA apparatus	Bio kit/ Spain
Eppendorf tubes 1.5 ml (microcentrifuge tubes)	Bioneer / Korea
Flasks, beakers and cylinders	Hirschman/Germany
Gel documentation system	bioneer/ Korea
Gloves and mask	Broche /China
High speed cold centrifuge	Eppendorf /Germany
Micropipettes0.5-10, 5-50, 100-1000µl	Eppendorf/ Germany
Plastic EDTA tubes 2.5 ml	AFCO /Jordan
Refrigerator	Concord / Lebanon
Rotator shaker	CYAN/ Belgium
Sensitive balance	Sartorius /Germany
Serum tube 5 mL	Jiangsu /China
Thermocycler (Gradient) PCR	Agilent technology surecycler8800 /United Kingdom
Shaker water bath	kottermann/ Germany
UV-Transilluminator	Cleaver scientific/ UK
Vortex mixer	Inc /china

2.1.2. Chemicals and biological materials

Table (2-2) the chemicals and biological materials with their companies and countries of origin used in this study.

Chemicals & biological materials	Manufacturer and Origin
Agarose	BHD /Canada
Deionize Distilled Water	Sigma/USA
Ethanol (96%)	BDH /England
Ladder 100bp	Biolab/Korea
Ethidium bromide	Sigma /USA
Free nucleases water	Bioneer /Korea
TE buffer	Promega /USA
Tris-Borate-EDTA Buffer (TBE buffer) 10x	Promega /USA

2.1.3. Commercial kits.

Table (2-3) the kits used in this study with their companies and countries of origin.

No.	Kits	Company	Country
1	**Toxo -Latex agglutination test**	**SPINREACT**	**Spain**
	Latex (Latex particles coated with soluble *T. gondii* antigen, pH 7.5 preservative)		
	Control + (animals serum with an antibody anti-toxoplasma concentration >4 Iu/ml)		
	Control- (animal serum preservative)		
2	**Anti Toxoplasma (IgG& IgM)**	**Bioactive**	**Germany**
	Micro titer wells		

	enzyme conjugate reagent		
	sample diluent		
	negative control, cut- off and positive control (lgM)		
	calibrator (0,1,2,3,4) for (lgG)		
	wash buffer concentrate (20x)		
	TMB reagent and stop solution (1 N HCL)		
3	**Primer**	**Alpha-DNA**	**Canada**
4	**Master Mix 100 reaction 2X**	**Bio-lab**	**Korea**
5	**Genomic DNA Extraction Kit (Blood, Tissue)**	**Favorgen**	**Korea**
	Collection Tube(200Pcs)		
	Elution Buffer(30m L)		
	Elution Tube(100Pcs)		
	FATG Mini Column(100Pcs) for tissue		
	FATG1 Buffer(30 mL) for tissue		
	FATG2 Buffer(30 mL) for tissue		
	FABG buffer(30 mL) for blood		
	FABG Mini Column(100Pcs)] for blood		
	Micropestle (100Pcs)		
	Wash Buffer (20m L)		
	W1 Buffer(44m L)		
	Proteinase K(11mg *2m l)		

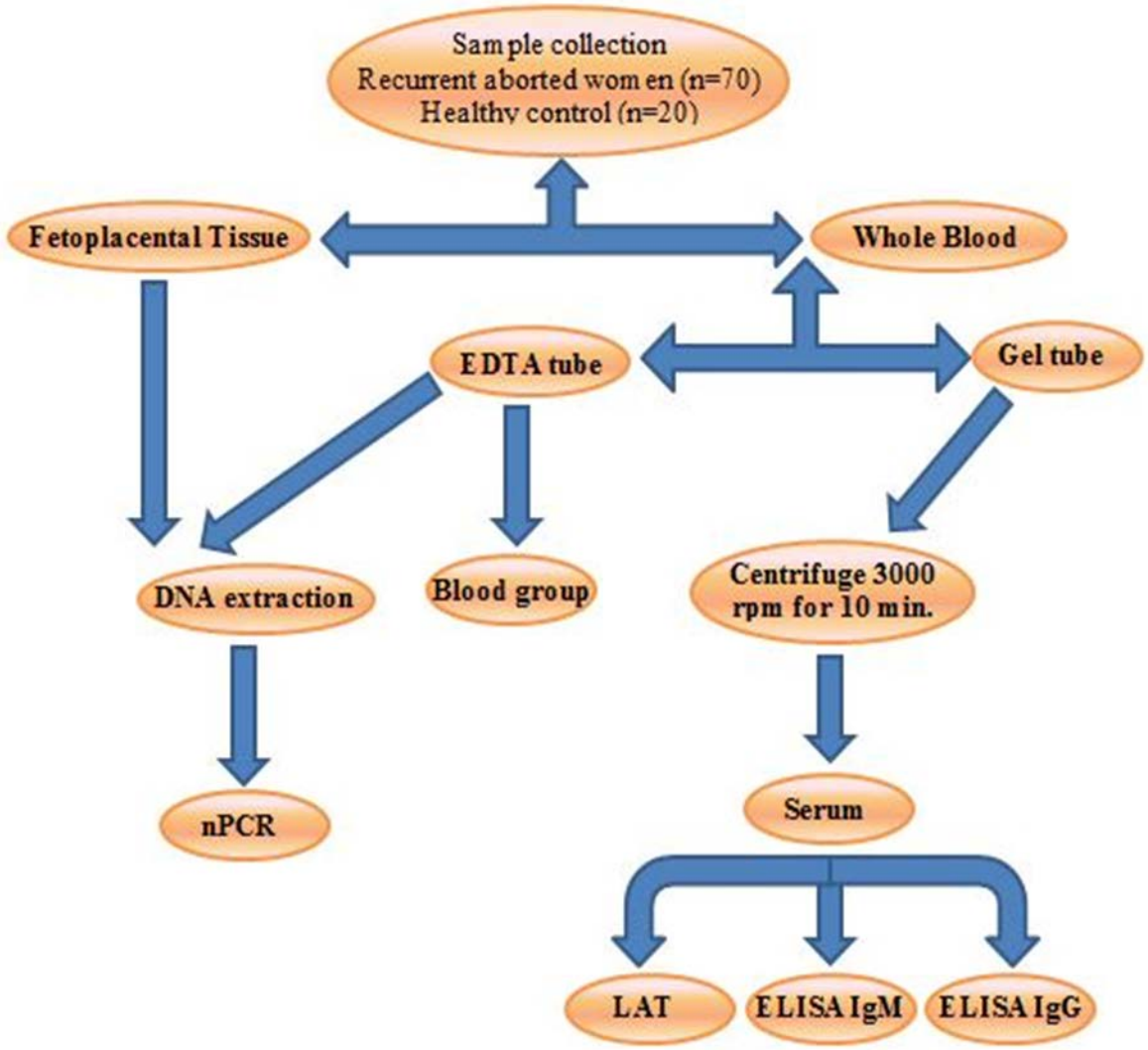
2.1.4. The Primer

Table (2-4): primer with their sequences used in this study and obtained from Alpha DNA (Mousavi *et al.*,2016).

Primer	Sequence (5' – 3')	Product size (bp)
Toxo-B1	F1: 5′-TCAAGCAGCGTATTGTCGAG	287
	R1: 5′-CCGCAGCGACTTCTATCTCT	
	F2: 5′-GGAACTGCATCCGTTCATGAG	193
	R2: 5′-TCTTTAAAGCGTTCGTGGTC	

F: Forward primer R: Reverse primer

Study design



2.2.The Patients and Controls:

The present study was conducted on 70 aborted women who suffer from recurrent aborted and 20 healthy women as a control group that pregnancy with normal delivery without any history of aborted. Samples were collected from patients suspected infection by toxoplasmosis and a control group who attended AL-Zahraa Maternity and Child Teaching Hospital in Najaf Governorate from December-2017 to March 2018.They were (16-42) years old age.

Questionnaire sheet

Case No. :

Hospital :

Name :

Age :

Residence : urban rural

Education:

Occupation:

Number of abortions:

Week of abortion :

Blood group :

Date:

Figure (1): The questionnaire sheet of study cases

2.2.1 Sample collection:

2.2.1.1 Blood samples

Five ml of venous blood were drowned from vein of each suspected patient and control groups by using disposable syringes, 3 ml from this blood were collected in a sterile serum tube and left 30 minutes at room temperature to separate the serum which was collected into eppendrof - tube by micropipette and stored at -20 °C until for serological test (*Toxo* latex agglutination Test, Toxo IgG, IgM-ELISA Kit), the remaining 2 ml blood sample was used for DNA extraction.

2.2.1.2. Placental tissue:

placental tissue samples(2-5)grams were collected from 70 recurrent aborted women and 20 healthy delivery women , kept with normal saline in sterile plastic containers and transferred to the research laboratory of Microbiology Department in College of science- Kufa University with cooling conditions (ice bags) for DNA extraction.

2.3.The Diagnostic Methods:

2.3.1 Serological tests

2.3.1.1. Latex Agglutination Test(code No. 1201002)

2.3.1.1.1.Test Principle:

The *Toxo*-latex is a slide agglutination test for the qualitative and semi-quantitative detection of anti-toxoplasma antibodies Latex particles coated with soluble *Toxoplasma gondii* antigen are agglutinated when mix with samples containing antibodies anti-toxoplasma .

2.3.1.1.2.Procedure of LAT.

2.3.1.1.2.1.Qualitative method

1-The reagents and samples were allowed to reach room temperature. The sensitivity of the test may be reduce at low temperature.

2-The patient serum (50 µl) was placed into a slide black area.

3-The latex reagent was mixed one drop was added over each serum drop.

4- Both drops were mixed by used wooden stick and tilt the slide.

5-the result was observed the presence or absence of agglutination within a period no longer than 4 minutes.

2.3.1.1.2.2. Semi-quantitative method

1- two fold of serial dilutions of the serum sample was made 9 g/L saline.

2-proceed for each dilution as in the qualitative method.

2.3.1.1.2.3. Reading and interpretation

The presence or absence of visible agglutination was examined macroscopically. The presence of agglutination was indicating as an antibody concentration equal or greater than 4 Iu/ml.

2.3.2. Immunological tests

2.3.2.1. Toxoplasma IgG Enzyme Linked Immune Sorbent Assay(Code No.TOXG01)

2.3.2.1.1. Principle of assay (lgG)

The Toxo-IgG01 – toxoplasma kit is based on ELISA technique. In the assay , calibrator and unknowns are incubated in The microtitration wells coated with purified and inactivated *T gondii* antigens.after incubation and washing , the wells are treated with the conjugated ,composed of anti-human lgG antibodes labeled with peroxidase .after a second incubation and washing step , the wells are incubated with the substrate tetramethylbenzidine (TMP).An acidic stopping solution is then added and the degree of enzymatic tumover of substrate is determined by wavelength absorbance measurement at 450nm.the absorbance measured is directly proportional to the concentration of anti-gondii antibody present.

2.3.2.1.2. Procedure (lgG)

It was done according to manufacturer's instructions as the following.

1- Marked microtitration method was applied.

2- The serum sample was diluted 1:101 by transferring 10 µL of serum sample in to plain tube contain 1 ml of sample diluent .

3- The mixture was pipetted, and 100 µl was of each diluted sample was transferred to and to appropriates wells.

4- The plate was incubated for 30 minute at 37 ℃.

5- The mixture was aspirated and each well was washed four (4) times for (30) seconds with washer solution.

6- One hundred microliters of enzyme labeled antibody was added to the each well.

7- The plate was incubated for 30 minute at 37 ℃.

8- The mixture was aspirated and each well was washed four (4) times for 30 seconds with washer solution

9- One hundred microliters of tetramethylbenzidine (TMP) chromogen solution was distributed to each well by using pipette.

10- The plate was incubated for 15 minute at room temperature.

11- One hundred microliters of stopping solution (1 N HCL). was added to each well using a pipette.

12- The plate was readed at absorbance 450nm within 30 minute.

2.3.2.2. Toxoplasma IgM Enzyme Linked Immune sorbent Assay(Code No. TOXG02)

2.3.2.2.1. Principle of assay (lgM)

The toxoplasma lgM assay is based on the principle of the capture of these immunoglobulin and subsequent identification of those, which are specific making use of their ability to bind an antigen conjugated to peroxidase. The capture is performed using monoclonal antibodies bound to the solid phase (microtitration strips). The antigen is composed of purified and inactivated *Toxoplasma gondii* antigen.

2.3.2.2.2. Procedure IgM

1- Marked microtitration method was applied.

2- The serum sample was diluted 1:101 by transferring 10 µL of serum sample in to plain tube contain 1 ml of sample diluent.

3- One hundred µl was pipetted of each diluted serum sample and ready to use controls to the appropriates wells .

4- The plate was incubated for 30 minute at 37 ºC.

5- The mixture was aspirated and each well was washed four (4) times for 30 seconds with washer solution.

6- One hundred µl was added of Toxo- horseredish peroxidase (HRP) conjugate into each well.

7- The plate was incubated for 30 minute at 37 ºC.

8- The mixture was aspirated and each well was washed four (4) times for 30 seconds with washer solution

9- One hundred µl was added of TMP chromogen solution to each well using a pipette .

10- The plate was incubated for 15 minute at room temperature ,avoid exposure to direct sun light .

11- One hundred microliters of stopping solution (HCL). Was added to each well using a pipette.

12- The plate was readed at absorbance 450nm within 30 minute.

2.4. Molecular level detection

2.4.1. Polymerase chain reaction (PCR)

The Polymerase chain reaction assay was used for detecting and differentiating the serotyping of *T. gondii* isolates .

2.4.2. Genomic DNA extraction / Blood Protocol (Code No. ABGK001-1).

Genomic DNA of *T.gondii* isolate was extracted by using the Genomic DNA .

1- Two ml of blood sample was collected in EDTA tubes.

2- Two hundred microliters of blood were added to the 1.5 ml micro centrifuge tube.

3- twenty µl of proteinase K was added, after that , 200 µl of Favorgen Blood Genomic (FABG) Buffer, and 100 µl of red blood cell lysis buffer was added and mix thoroughly by pulse-vortexing.

4- The tubes were incubated for 15 minutes at water bath at 60 ºC to lyses the red blood cells

5- During incubation, the sample was vortex every 3-5 minutes.

6- Briefly the tubes was spin to remove drops from the inside of the lid.

7- After that, 200 µl of ethanol was added (96-100%) to the tube, then mix thoroughly by pulse-vortex for 10 second.

8- Briefly the tubes was spin to remove drops from the inside of the lid.

9- FABG Mini column collection tube was prepared, and the mixture was transfer the mixture (including any precipitate) carefully to the FABG Mini column .

10- The tube was centrifuged at 6,000×g for 1 min, then FABG Mini column tubes was transferred to a new collection tube.

11- Four hundred microliter of W1 Buffer was added to FABG Mini column and centrifuge at full speed (18,000 ×g) for (30) second then discard was the flow-through.

12-750 microliter of wash buffer was added to the FABG Mini column and centrifuge at full speed for 30 sec, then discard the flow-through.

13- Again the tubes were centrifuged at full speed for an additional (3) minutes.

14- the FABG Mini column tubes was placed to a elution tube.

15- About 50-200 µl was added of heated Elution Buffer or ddH2O (pH7.5-9.0) to the membrane center of FABG Mini column. stand FABG Mini column for 3 minutes.

16- The tubes were centrifuge at full speed for 1 minute to elute total

DNA in the sample.

17- stored total DNA at -20 ºC.

2.4.3. Genomic DNA Kit / Tissue Protocol (Code No. FATGK001-1)

1-The three piece of tissue was cut up to mg (or 0.5 cm) then transfer to a 1.5 ml microcentrifuge tube provided with kit micro pestle to grind the tissue sample

2- Two hundred microliters of FATG1(Favorgen Tissue Genomic) buffer was added and mix well by pipett tip.

3-Then 20 microliters of proteinase K was added to sample mixture, mix thoroughly by vortexing.

4-The tubes were incubated for 1-3h at 60 ºC until tissue is lysed completely, vortexing was performed occasionally during incubation.

5- Two hundred microliters of FATG 2 Buffer was added to sample mixture, then mix thoroughly by pulse-vortexing and incubated for 10 minutes at 70 ºC.

6- Two hundred microliters of ethanol (96%) was added to the sample mixture, mix thoroughly by pules-vortexing.

7-The tube was spine to removed drops from the inside the lid.

8-placed a FATG Mini column in a collection tube. transfer the mixture carefully to the FATG Mini column. Centrifuge at full

(~ 18,000×g) for 1 min then place the FATG Mini column to a new collection tube.

9- four hundred microliters of W1 Buffer were added to FATG Mini column, then centrifuged at full speed for 1 min then discard flow-through.

10-750 microliters of wash Buffer was added to the FATG Mini column, then centrifuge at full speed for 1 min then discard flow-through.

11-Again the tubes were centrifuge at full speed of an additional 3 min to dry the column.

12- One hundred microliters of preheated Elution Buffer or ddH20 (pH 7.5 - 9.0) was added to the membrane of the FATG Mini column tubes.

13-The tubes was centrifuge at full speed for 2 min to elute DNA.

14-The extracted DNA was stored at -20 ºC

2.4.4.-nested Polymerase chain reaction(nPCR) (Mousavi *et al.*, 2016).

2.4.5. DNA Amplification

The infections of *T. gondii* confirmed by PCR amplification of *B1* gene using specific gene primers as following:

1- Ten microliters of master mix was transferred to 0.2 ml PCR tube.
2- One µl of each primer (forward and reverse) has been added to PCR tube.
3- Five µl of purified DNA (extracted from blood or tissue) has been added to PCR tube.
4- Three µl of deionized water was added to completed a total reaction volume.
5- To homogenize the contents, the mixture was mix briefly by centrifugation for 3000 x g for 10 seconds.
6- Twenty µl for each amplification reaction was placed in the thermal cycler and amplified as mentioned in table (2-5).

7- Finally, the second run was the same nPCR program in above point except the annealing temp. was 55 C˚ with 35 cycle.

The Table (2-5)n PCR program

Step1	Temp. (C˚)	Time	Cycle
Initial denaturation	94	5 min.	1
Denaturation	94	1 min.	28
Annealing	57	1 min.	
Extention	72	1 min.	
Final extention	72	5 min.	1

The expected length of the PCR product was 193 bp

2.4.6. Gel electrophoresis

All PCR products of detection and serotyping genes were analyzed by loading in agarose gel in different percent as described as follows:

1- Agarose gel was prepared by dissolving 1.5 g of agarose in 100 ml of TBE (1X ,pH 8.0).

2-The agarose gel solution was dissolved in water bath at 100 °C for 15 minutes and left to cool 50°C. Then 4µL of ethidium bromide stain were added into the agarose gel solution.

3- The agarose gel solution was poured in to a tray after fixing the comb in a proper position and left to solidify for 15 minutes at room temperature, then the comb was removed carefully from the tray and 5 µl of PCR product were added into each comb well and 5µl of (100bp Ladder) in one well.

4- The gel tray was fixed in the electrophoresis chamber and fill by 1X TBE buffer. Then an electric current was performed at 80 volts and 70 AM for 1hours.

5- PCR products (bands) were visualized by using gel documentary system.

2-5 Statistical Analysis

The data were analyzed by SPSS (version)22. A chi-square test compared the seroprevalence values with the gene *B1* of *T. gondii*. Confidential intervals at 95% and $P < 0.05$ were considered levels of significance (Aljanaby and Alhasnawi, 2017).

Chapter Three
Results and discussion

3.The results and discussion

The current study was conducted at Al-Najaf province from December 2017 to March 2018. 70 recurrent aborted women suspected infection with *T.gondii* recruited to AL-zahraa Maternity and Children teaching hospital and 20 healthy women with normal delivery without any history of abortion as comparison group ,who admitted to the same hospital.

The table (3-1) show the relationship among Latex agglutination test, Enzyme linked immune sorbent assay (lgG, lgM), and nested polymerase chain reaction for (blood and tissue) in toxoplasmosis detection.

For LAT, there is a significant relationship between recurrent aborted women and healthy control (p = 0.0001 value), 44(62.9%) out of 70 were positive by LAT and 20(0%) for recurrent aborted women and healthy control respectively.

Table (3-1) the percentage of toxoplasmosis according to Latex agglutination test, ELISA (IgG , IgM), n PCR tissue and nPCR blood in recurrent aborted women by *Toxoplasma gondii* .

Variables		Patient		*P*-value (Sig.)
		No. of sample	%	
Latex agglutination Test	Positive	44	62.9%	0.0001 (S)
	Negative	26	37.1%	
ELISA IgG	Positive	33	47.1%	0.0001 (S)
	Negative	37	52.9%	
ELISA lgM	Positive	0	0.0%	0.0(NS)
	Negative	70	100.0%	
nPCR tissue	Positive	24	34.3%	0.002 (S)
	Negative	46	65.7%	
nPCR blood	Positive	12	17.1%	0.047 (S)
	Negative	58	82.9%	

P value was ≤ 0.05 considered significant

Latex agglutination test was used for general detection of toxoplasmosis because it is comparatively simple, inexpensive and has provided an effective method for routine serological analyzes of the antibodies to toxoplasmosis (Hasson, 2004).

This can be explained by the constant exposure of women to the risk factors of *T. gondii* infection through their routine household tasks such as minced meat products, raw and unwashed vegetables and fruits, municipal drinking from polluted reservoirs, In addition to the spread of stray cats that play a key role in the distribution of infection (Cook *et al*. 2000).

This may be due to differences in the availability of optimal environmental conditions for survival and spread of parasites in addition to the presence of

more than one risk factor affects the incidence of toxoplasmosis as people's habits and health conditions (Han *et al.* 2008).

The result of this study was agree with Al-Khaffaf (2001) in Mosul was 69.2% , Abbas (2002) in Baghdad was 60.21% and Aaiz, (2010) in Najaf was (59.9%) respectively .

The result of this study disagree with Othman (2004) in Kirkuk was (36%); Al-Kaysi and Ali (2010) in Baghdad was (22%),Al-Simani (2000) in Mosul who found the rate of toxoplasma seropositivity (39.53%) in pregnant women by using LAT.

For ELISA lgG, the positive cases were 33 (47.1%) for recurrent aborted women (*p* value= 0.0001)This results were statistically significant.

The chronic toxoplasmosis, most probably is a significant cause of repeated abortion and it may be due to the reactivation of chronic infection of the uterus, when no parasitic medication is given after the first abortion, causing rupture of the tissue cyst as a result of the enlargement in the size of the uterus during pregnancy causing liberation of the organisms and fetal infection and damage leading to pregnancy wastage (Hamad and Kadir, 2014).

The result of this study was agreement with study in Al- Najaf Lalia *et al*. (2004), Al-Khafajy (2004) and Al-Kalaby (2008) in Al-Najaf found the toxoplasma IgG test was positive in (48%) ,Aaiz (2010) reported that IgG ELISA test was positive in (46.75%) of total cases, in Libyan, (Mousa *et al*., 2011) reported that IgG-ELISA test was positive in (44.8%) of total cases , in Qadisiyah (Hadeel *et al*., 2016) reported that IgG ELISA test was positive in (44%) of total cases.

The result of this study was disagreement with El-awady *et al*. (2000)in Al-Najaf recorded that *Toxoplasma* IgG antibody was 93.1%. It was also who lower than the results of Jumaian (2005) in Jordan was IgG 97.5 and Al-

jorany (2011) in Al-Najaf observed that the *Toxoplasma* IgG was 69 (98.5%). Also this result was no compatible with others study such as Tabbara and Saleh (2005) in Bahrain who showed that the *Toxoplasma* seropositivity rate was 21.8% for IgG.

Mahdi *et al*. (2006) in Basrah pointed out that the percentage of infection was 7% IgG, Salih (2010) in Al-Najaf showed that the percentage of IgG was 30.76% and Mohammed (2011) showed 36% *Toxoplasma* positive IgG, Dory (2011) in the Salah Aden City showed that the percentage of IgG was 26.1%,Harith and Ban (2012) in Baghdad demonstrated that there was a high prevalence of IgG 31.70% , Al-Hindi and Lubbad (2009) in Gaza by found that the prevalence of *Toxoplasma* IgG was 17.9% among Palestanian women and (Al-Hamdani and Mahdi 1997) mentioned that Toxoplasma IgG positive rate was 18.5% .However, The differences may be related to different commercial kits ,with different washing solution, buffering solution and standard (Noori and Mahdi, 2016). Moreover the difference may attributed to social and cultural habits, geographic factors, climate, transmission route and age differences, and this interpretations come nearly similar to that of (Cantos *et al*., 2000).

For ELISA lgM, the positive cases were 0 (0.0%) for recurrent aborted women (*p* value=0.0).This results were statistically non-significant.

The current study not detect IgM in recurrent aborted women indeed, this study involved a specific group of women, so it was difficult to generalize the current seroprevalence into all populations, that may not reflect the real size of problem in our community (Mohammed, 2011).

The IgM antibodies was lower than cut-off value that may be attributed to the nature of infection in which most of infected women do not attending the hospital during the acute stage until abortion occur and the proof of this fact

Aaiz (2010),as well as, the life Spin of lgM less than 14 days (Lewis *et al.*, 2008).

The results of this study were agreement with modern studies (Firouz *et al*. 2014; Modrek *et al.* 2014 and Anubhuti *et al.*, 2015) reported that the seropositivily of IgM was (0%).

The result of this study was disagreement with other researchers from different parts of the world such as(Rai *et al.*, 1998) in Nepalese 345 women found only 3% were positive IgM ; (Marcolino *et al*., 2000) in India 300 pregnant women were 3% had positive Toxoplasma IgM, Al-Quraishi (2001) in Saudi Arabia and Tabara and Saleh (2005) in Bahrain reported a prevalence of IgM were 5%;(Razzak *et al.*, 2005) in Duhok who recorded 3 women (1%) out of the 310 patients were positive IgM ; (Kifah *et al.*, 2006) in Najaf province recorded 4.25% positive IgM; (Abu-Mahdi *et al*., 2008) found that patients with IgM positive was 4.4%, Akyar I (2011) in Turkey reported that the seropositivity of *T. gondii* was 1.34% for IgM and Dory (2011) in the Salah Aden City showed IgM was 3.1%.

For nPCR (blood samples) the positive cases were 12(17.1%) for recurrent aborted women (*p* value = 0.047).This results were statistically significant.

For nPCR (tissue samples) the positive cases were 24(52.9%) for recurrent aborted women (*p* value =0.002).This results were statistically significant.

Particularly, PCR is more specific and more sensitive technique that widely used generally in research and specifically in routine work (Hafid *et al*., 1995).Today PCR has successfully been used for diagnosis cerebral, congenital, ocular and disseminated toxoplasmosis (Bourdin *et al*., 2014). In time of many limitations of serological methods in detection antitoxoplasma antibodies may not present in early infection, that may not arise during reactivation of cystic forms of the parasite (Dubey *et al*., 2003).

The differences of percentages that recorded in the current study and other studies may be attributed to the different origins of samples used samples (blood, amniotic fluid, placenta tissue, etc.) and this interpretation was stated by (Marie *et al.*, 1999) and (Al-Kalaby, 2008) when they recorded a significant differences among different used samples or due to immunocompetent or immunocompromised status of patients (Israelski and Remington, 1993).

The detection of the parasite DNA by PCR has considerably improved diagnosis, particularly the prenatal diagnosis of congenital disease (Morelle *et al*., 2012). Salotra & Singh(2006) reported that PCR could detect parasitaemia a few weeks prior to the appearance of any clinical signs or symptoms .

Many studies concluded that ,the lower number and percentage positive cases by using blood samples may be due to small volume of blood specimens or DNA molecules of *T. gondii* which used as source of *T. gondii* DNA in compared to whole blood in the body of human and small number of parasite in peripheral blood as well as due to many inhibiter materials in the blood lead to inhibit the PCR reaction, such as hemoglobin, Lactoferrin, immunoglobulin G and haem (Ajzenberg *et al.*, 2009).

Also may be due to few quantity of *Toxoplasma* DNA may be extracted from clinical samples (Khan *et al.*, 2005;Nowakowska *et al.*, 2006).

The result of this study was agreement with Al-Addlan (2007) who reported that 17.65% of the total blood samples were positive.

The results of this study were disagreement with studies of Aaiz (2010), Al- Kalaby (2008) and Mohammed (2011)which conducted on blood samples who founded that the percentage of infection by nPCR was 58.6%, 83.3% and 56% respectively. (El- awady *et al.*, 2000) who found that 96.6% when used by nPCR. (Okay *et al*., 2009) who found that 63.49% of tested blood samples from Brazilian women was positive when used by nPCR .

This result of this study was disagreement with(Thomas *et al*., 2004) mentioned that the positive cases by nPCR (tissue samples) were 75%.

The detection of toxoplasmosis by using specific primer *B1*gene for nPCR technique revealed highly specific in magnification of toxoplasma DNA and fruitful in the detection of *T. gondii* DNA from tissue sample more than blood sample of aborted women infected with *T. gondii* (figure 3-1,3-2).

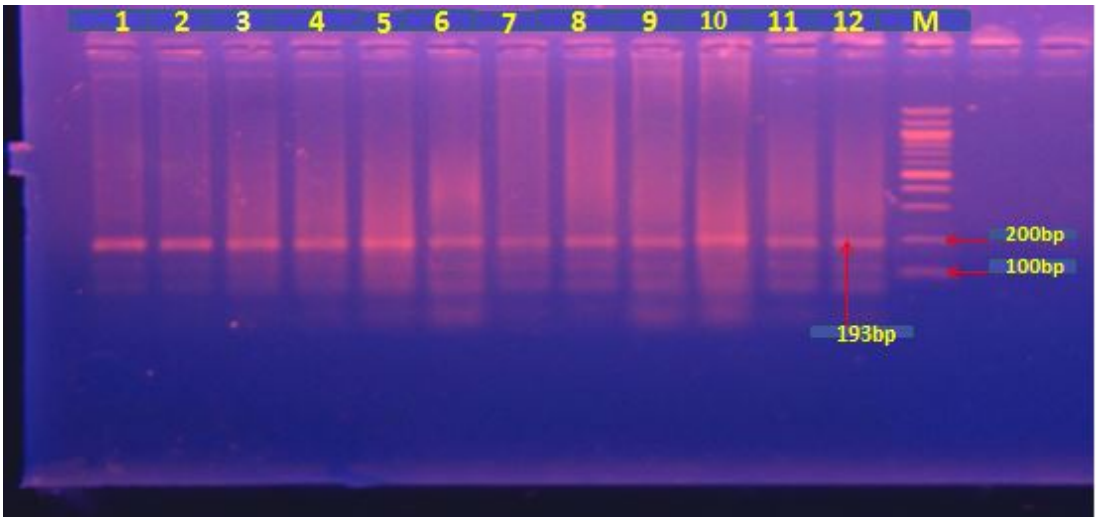


Figure (3-1) Amplification of toxo*B*1 gene of *Toxoplasma gondii* DNA from the blood of recurrent aborted women. Lane-M, molecular weight marker (100 bp ladder, 100 to1200bp), Lanes 1-12 positive samples at 193bp. Running conditions: Agarose gel (1.5%), 80 volt for 60 Minutes, stained with ethidium bromide.

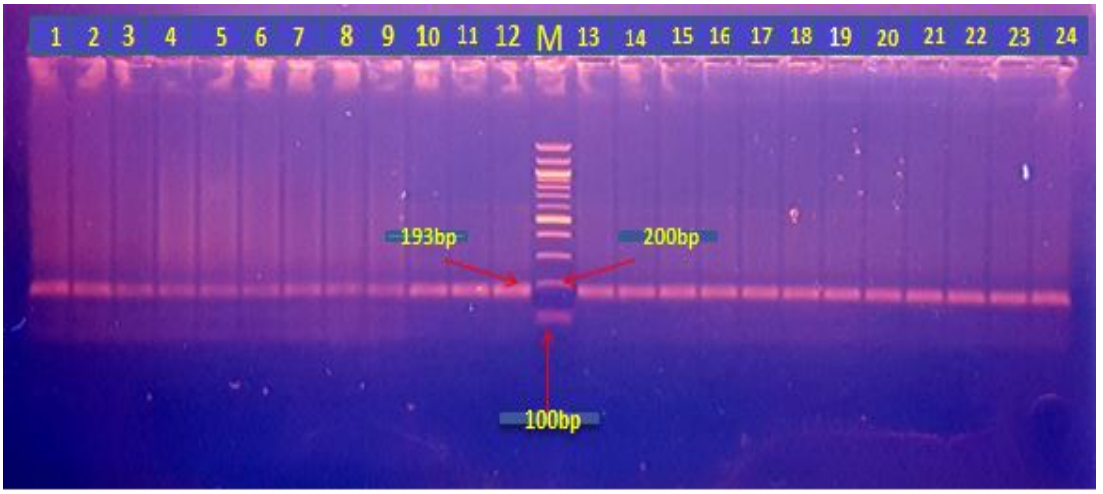


Figure (3-2) Amplification of toxo *B1 gene* of *Toxoplasma gondii* DNA from the tissue of recurrent aborted women. Lane-M, molecular weight marker (100 bp ladder, 100 to1200pb), Lanes 1-24 positive samples at 193bp. Running conditions: Agarose gel (1.5%), 80 volt for 60 Minutes, stained with ethidium bromide.

Also may be due to few quantity of *Toxoplasma* DNA may be extracted from clinical samples (Khan *et al.* 2005; Nowakowska *et al.* 2006).

The highest sensitivity of *B1* primers may be because that *B1* primer was specific to strain of *T. gondii* found in Iraq (Colin *et al*., 2000). Results by Hurtado *et al*.(2001) , Adriana Calderaro *et al*.(2006) and (Elisabeth *et al.*, 2004). Agreed with results of the current study which found *B1* gene and 18S have high specificity and sensitivity, therefore, the have been used in diagnosis of *T. gondii* parasites by PCR technique (Hadi & Al-Hadrawy 2015).

The table (3-2) shows the comparative between the three tests LAT, ELISA (lgG), and nPCR (blood and tissue) based on age group revealed the higher number and percentage of infection in age(21-27) years of three tests were 20(45.49%),12(36.36%), 5(41.66%) and 10(41.66%) respectively. However the results were non-significant (*p* value >0.05).

Table (3-2) the percentage of infection with toxoplasmosis based on age group by using Latex agglutination test, ELISA (IgG), nPCR blood and nPCR tissue .

Age group	Number of sample	Number of positive by LAT(%)	Number of positive by lgG (%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue (%)
<=20	10	8(18.1)	5(15.15)	1(8.33)	3(15.5)
21-27	30	20(45.49)	12(36.36)	5(41.66)	10(41.66)
28-35	18	9(20.49)	9(27.27)	4(33.33)	8(33.33)
Up 36	12	7(15.9)	7(21.2)	2(16.66)	3(12.5)
P-value		>0.05	>0.05	>0.05	>0.05

P value was ≤ 0.05 considered significant.

The current age group (21-27) year may be represents an optimum period of fertility, thus, this critical period of women's life has higher chances for activation of latent infection of *T. gondii* that can be transmitted vertically to the fetus, which was considered as one cause of abortion as mentioned by (Remington *et al*., 2001).

The second decay of age (21-27) represents a mother's stage and the prenatal detection of antibodies against *T. gondii* in pregnant women, it was critical with regard to the management of serious congenital complication including abortion (Han *et al.*, 2008).

In line with several studies conducted in Iraq, the current result was agreement with Al-Doski (2000) ; Hasson (2004); Al-Ani (2004); Al-Addlan (2007); Al-Rubaia (2008);Mohammed (2008) in Hilla ; Aaiz (2010) and Mohammed (2011). Also the result agrees to study of reported by Alghamdi *et al*. (2016) in Saudi Arabia, Coelho *et al.* (2003) in Brazil, Al-Hindi &Lubbad (2009) in Palestine and Shin *et al.* (2009) in Korea.

The result of this study disagreement with Al-Hamdani and Al-Mahdi (1996) who found that the seropositivity to *Toxoplasma gondii* significantly increased with age group (35-45) years. Also disagreement with Ageel (2003) and Kadir *et al*. (2011) who found high seropositivily in age (25-35) years.

The table (3-3) shows the comparative between the three tests LAT, ELISA (lgG), and nPCR (blood and tissue) based on levels of education revealed the higher number and percentage in don't read and write of three techniques were 26(59.09%), 22(66.66%), 6(50.0%) and 12(50.0%) respectively. However the results were non-significant (*p* value >0.05).

Table (3-3) the percentage positivity of toxoplasmosis based on Education by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Education	Number of sample	Number of positive by LAT(%)	Number of positive by lgG(%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue(%)
Don't read and write	43	26(59.09)	22(66.66)	6(50)	12(50)
Primary school	14	11(25)	7(21.21)	4(33.33)	4(16.66)
Middle school	4	1(2.27)	0(0.0)	0(00.0)	2(8.33)
Preparatory School	9	6(13.63)	4(12.12)	2(16.66)	6(25)
P-value		>0.05	>0.05	>0.05	>0.05

P value was ≤ 0.05 considered significant.

The results of study revealed high incidence of toxoplasmosis among low level education this result may be due to that the legal obligation of education was not found in my country and another important causes statistical association was not found between *T. gondii* seroprevalence and the education level of the women but the disease is more prevalent among women with school education and illiterate patients almost in all the tests and lower seroprevalence was present between women had college education because increased knowledge results in wareness, which consequently results in changes in risky behavior and decline in infection rates (Ertug *et al.*,2005).

The result of this study was constant with Al-Griari (2007) in Diyala, Ertug *et al*. (2005) and Sert *et al*. (2007) in Turkey, While Mohammed (2012) found the occurrence of the disease was higher among uneducated people than educated ones.

The result of this study was not constant with Jones *et al*. (2001) who reported that the seroprevalence was higher among those with education below college level in United States. While Nsagha *et al.(*2011) who reported that the seroprevalence was higher among middle school by about(55.5%).

The table (3-4) shows the comparative between the three tests LAT, ELISA (lgG), and nPCR (blood and tissue) based on blood group revealed the number and percentage were higher in blood group (B) by nPCR for tissue 12(50.0%) and the lower number and percentage were in blood group O 1(4.16%). In spite of the results were non-significant(*p* value >0.05).

Table (3-4) the percentage positivity of toxoplasmosis based on Blood group by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Blood group	Number of sample	Number of positive by LAT (%)	Number of positive by lgG(%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue (%)
A	29	21(47.72)	15(45.45)	3(25)	8(33.33)
B	28	16(36.36)	13(39.39)	7(58.33)	12(50)
AB	10	5(11.36)	4(12.12)	2(16.66)	3(12.5)
O	3	2(4.54)	1(3.03)	0(0.0)	1(4.16)
p-value		>0.05	>0.05	>0.05	>0.05

P- value was ≤ 0.05 considered significant.

This results discussed depend on sensitivity and specificity of the nPCR of tissue samples, The A , B and O blood group system are determine by the presence or absence of A and B carbohydrate antigens on the surface of red blood groups (Hakomori, 1999).

Certainly, the molecules that define ABO blood group phenotypes consist of carbohydrate that are present in the glycoproteins structures and expressed in red blood cells and other tissues (Schenkel ,Brunner 2000).

The adherence mechanism of micro-organisms to mucous membranes of hosts is not totally clear, but it is likely that glyco-conjugates of the ABO group system are involved in this process , It is of interest that the present study revealed an association between blood group system and *Toxoplasma* infection with highest prevalence among blood (Henry, 2001).

The result of this study was agreement with Al-Shikhly (2010) in Baghdad who showed the higher prevalence was occurred in female with (B)blood group. Zamorano *et al*. 1998; Movayedi, 2000 and Modrek *et al*. 2014 in Iran they reported ,the high prevalence of toxoplasmosis was occurred in blood group (B).

The result of this study was disagreement with study conducted in Baghdad Al-Saadii (2013) who reported that high prevalence of *Toxoplasma* infection was in blood group (O). (Obaid, 2014) who reported that the prevalence was higher among blood group (A) and (AB). Chiang *et al*. (2012) who reported that in Taiwanese people the seroprevalence was higher among of blood group AB than people of blood group O ,however, the difference was not statistically significant.

The Table (3-5) shows the comparative between the three test LAT, ELISA (lgG), and nPCR (blood and tissue) based on occupation revealed the higher number and percentage in Housewife of three tests were 38(86.36%), 30(90.91%), 11(91.66%), 20(83.33%) respectively. However, the results were non-significant (*p* value >0.05).

Table (3-5) the percentage positivity of toxoplasmosis based on Occupation by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Occupation	Number of sample	Number of positive by LAT(%)	Number of positive by lgG (%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue (%)
Housewife	61	38(86.36)	30(90.91)	11(91.66)	20(83.33)
Employee	9	6(13.63)	3(9.09)	1(8.33)	4(16.66)
p-value		>0.05	>0.05	>0.05	>0.05

P- value was ≤ 0.05 considered significant.

The higher rate of infection among housewives than Employee might be related to housewives being in direct contact with infection through handling and preparing of food (contaminated meat and vegetables) in addition to cleaning of house garden contaminated with cat feces, the majority of women in this study were housewife and few of them are employee(Kadir *et al*., 2011).

The results of this study were agreement with other studies like A'aiz (2010) in Najaf; Hamad and Kadir (2013) in Erbil; Al Se´adawy (2010)in Al Muthana ; Aqeely (2014)in Saudi Arabia and Esquivel (2006) in Mexico they are said that, the high percentages of toxoplasmosis were recorded among housewives.

The results of this study were disagreement with Al-Kalaby (2008) reported , the employed were more affected with toxoplasmosis. Also Mwambe (2013) said that, business women and employed pregnant ,officials women had higher infection rates with *T. gondii* than housewife.

The table (3-6) shows the comparative between the three test LAT, ELISA (lgG), and nPCR for blood ,tissue based on residency revealed the higher number and percentage in urban of three test were

31(70.45%),21(63.63%),8(66.66%), 18(75.0%)respectively. In spite of the results were non-significant (*p* value >0.05).

Table (3-6) the percentage positivity of toxoplasmosis based on residency by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Residency	Number of sample	Number of positive by LAT(%)	Number of positive by lgG (%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue(%)
Urban	47	31(70.45)	21(63.63)	8(66.66)	18(75)
Rural	23	13(29.54)	12(36.36)	4(33.33)	6(25)
p-value		>0.05	>0.05	>0.05	>0.05

P -value was ≤ 0.05 considered significant.

The probable causes of the prevalence of disease in urban areas were attributed to high distribution of stray cats that will contaminate vegetables where the washing and removal of oocyst may not be accomplished due to not enough water supply for some areas in the city (Elsheikha, 2008).

The other possible causes were due to high utilization of meat, that may be contaminated with tissue cysts or may be due to the dealing with a high number of cases in urban area compared with the rural area, all these factors may play a role in the high incidence among the urban population when compared with rural one (Kim *et al*., 2008).

In addition consumption of undercooked/partially cooked meat including Pastrami, luncheon, sausages, burgers, minced meats and similar foods (Amin *et al*., 2013).

The results of this study were agreement with Hasson (2004); Razzak (2005) and Al-Kalaby (2008) in Iraq they reported ,the high percentages of

toxoplasmosis were recorded among urban, Ertug (2005)in turkey said that, the high percentages of toxoplasmosis were recorded in urban.

The results of this study were disagreement with Several studies like Al-Griari (2007) in Diyala; Kadir and Khana (2009) in Sulaimani ; Ali (2008) in Kalar and A'aiz(2010) in Najaf they reported higher prevalence in rural regions in comparison with urban areas.

The Table (3-7) shows the relationship between the studied test LAT, ELISA (lgG), and nPCR (blood and tissue) based on Number of abortion revealed the higher number and percentage in (3-6 aborted) of three tests were 37(84.09%), 28(84.84%), 10(83.33%), 21(87.5%) respectively. In spite of the results were non-significant (*p* value >0.05).

Table (3-7) the percentage positivity of toxoplasmosis based on Number of abortion by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Number of abortion	Number of sample	Number of positive by LAT(%)	Number of positive by lgG (%)	Number of positive by nPCR blood(%)	Number of positive by nPCR tissue (%)
<=2	13	6(13.63)	3(9.09)	2(16.66)	3(12.5)
3-6	54	37(84.09)	28(84.84)	10(83.33)	21(87.5)
Up7	3	1(2.27)	2(6.06)	0(0.0)	0(0.0)
p-value		>0.05	>0.05	>0.05	>0.05

P- value was ≤ 0.05 considered significant.

The parasite remain in latent state and again when the mother become pregnant where her immunity suppressed due to certain physiological changes in the body that occur during pregnancy, the parasite will reactivated and become the cause of her next abortion unless the treatment is received(Jose and Jack 2008).

The results of this study were agreement with study of Al-Hamdani *et al*. (1996) referred they women with four or more abortions infected with *Toxoplasma gondii*. Hasson (2004) showed that, the number of infected women who were with three abortion.(Hamad and Kadir 2014) referred to that, the most cases were in women mainly who had three aborted cases.

The results of this study were disagreement with Al-Degali (1998) who observed that, the abortion for two times represented the higher percentage of positive cases, Al-Kalaby, (2008) showed that, the number of infected women who were with one abortion . KaremL (2007) and Al-Khashab (2009) where they found the highest rate of infection among women with single abortion. Was more than others a most recent study conducted in Diyala (Darweesh *et al*., 2018).

The table (3-8) shows the comparative between the three tests LAT, ELISA (lgG), and nPCR (blood and tissue) based on week of abortion revealed the higher number and percentage in (9-12) week of three tests were 36(81.81%), 25(75.75%),8(66.66),16(66.66) respectively, In spite of the results were non-significant (*p* value >0.05).

Table (3-8) the percentage positivity of toxoplasmosis based on week of abortion by using Latex agglutination test, ELISA (IgG), n PCR blood and nPCR tissue .

Week of abortion	Number of sample	Number of positive by LAT(%)	Number of positive by lgG (%)	Number of positive by nPCR blood (%)	Number of positive by nPCR tissue (%)
<=8	15	8(18.18)	8(24.24)	4(33.34)	8(33.34)
9-12	55	36(81.81)	25(75.76)	8(66.66)	16(66.66)
p-value		>0.05	>0.05	>0.05	>0.05

P -value was ≤ 0.05 considered significant.

This may be due to the fact that first trimester of the pregnancy is considered as a critical period in which the fetus is not well established in the uterus and it is threatened for abortion whenever the mother is expose to any risky factor such as reactivation of latent infection as *T. gondii* that result from immunosuppressant concomitant with pregnancy which can lead to placental infection and next placental insufficiency, with subsequent embryonic death (AL-Salihi and AL-Aaraji, 2011).

Persistence of encysted forms of *Toxoplasma* in chronically infected uteri and their subsequent rupture during nutrition of fetus through placenta leads to infection of the baby in the first trimester and often results in recurrent abortion(Jose and Jack 2008).

The results of this study were compatible with study of (Hacker *et al*., 2010; AL-Salihi & AL-Aaraji 2011 ; Kadum, 2013; Darweesh *et al*.,2018) whom reported that the higher incidence of abortion accrues in first trimester.

The results of this study was incompatible with (Mohammed, 2008; Ramsewak, 2008 ; Al-Khanak, 2009) they are reported that the higher incidence of abortion accrues in second trimester.

The specificity and Sensitivity of the nPCR tissue was more sensitive and specific than other tests by about (66,100) respectively (*p* value 0.02) in comparison with other methods nPCR from blood samples (83,100 non sig.) respectively, LAT (37,100) respectively and ELISA IgG (53,100) respectively (see figure 3-3)**.**

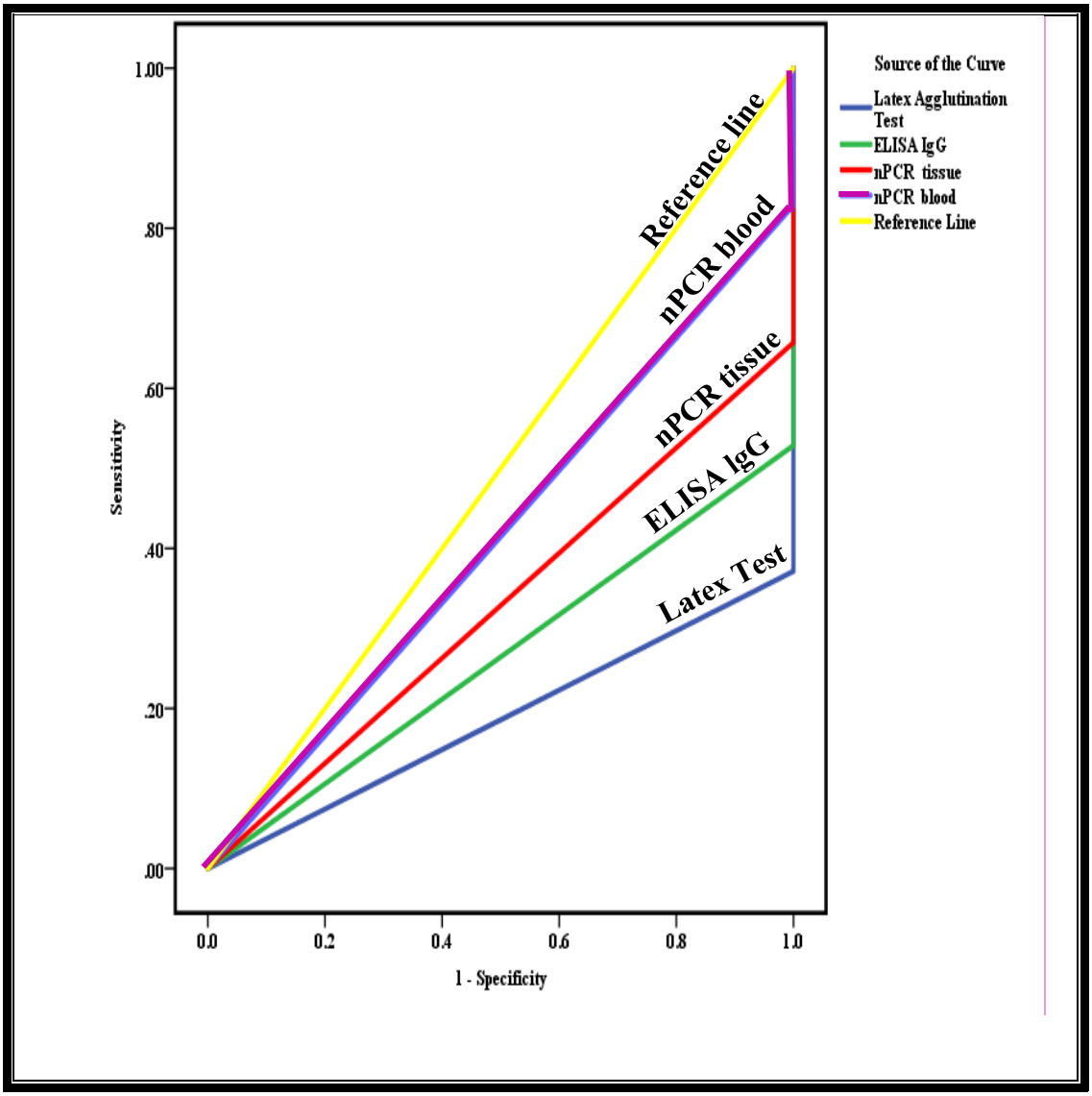


Figure (3-3) showed the receiver operating characteristic curve of Latex agglutination test, ELISA IgG, nPCR tissue and nPCR blood in abortion women by *Toxoplasma gondii* comparison with Reference Line.

$$\text{Specificity} = \frac{\text{Negative cases}}{(\text{False Positive cases} + \text{True Negative cases})} * 100$$

$$\text{Sensitivity} = \frac{\text{Positive cases}}{(\text{True Positive cases} + \text{False Negative cases})} * 100$$

The difference in the specificity and sensitivity between these two test LAT and ELISA may be due to the different antigenic epitopes being recognized by each test (Rigsby *et al.*, 2004) .

The sensitivity and specificity of the nPCR depend on multiple factors, such as the characteristics of the DNA sequence that is amplified, the DNA extraction protocol and The optimization of the reaction conditions (Bustin *et al*., 2009).

The sensitivity and specificity of a test cannot be used to estimate the probability of disease in a patient, but the parameters could be combined into one measure called the probability ratio which may be used in conjunction with disease prevalence to estimate an individual patient's probability of having disease (Lang and Secic 1997) ; (Akobeng AK, 2007).

The results of study conducted that nPCR assay from tissue and blood samples more sensitivity and specificity than ELISA IgG assay and LAT this may be due to delay or failure the body to produce antibodies or for presence some inhibitory substances such as Calmodulin, myosin, actin and tubulin intra cytoplasmic of *T. gondii* may be elucidate false positive results through serological diagnosis of parasite infections (Hadi & Al-Hadrawy 2015).

This result corresponded with the study by (Vujanić, 2012) and (Majeed and Hashoosh, 2014). That diagnosed *T. gondii* by serological and molecular test and they found PCR method was more sensitive and specific than IgG and IgM specific ELISA test.

A successful PCR technique in detection of parasite DNA in acute infection may belong to PCR assay not dependent on the viability of parasite which detected all the *T.gondii* dead and viable in peripheral blood of human *T. gondii* rapidly killed by the immune system but the DNA remains for some time in peripheral blood of human (Guy & Joynson, 1995).

Negative PCR of blood samples may be due to few number of *T. gondii* In the peripheral blood short remain time of parasitaemia or small size of blood sample which used to DNA is extracted compared to the total volume of blood in the human body and presence some inhibitory substance in human blood that may impede the reaction of PCR assay such as hemoglobin, haem, immunoglobulin G and Lactferrin (Vujanić, 2012).

The results of the present study corresponded with the study of (Vujanić, 2012) which that reported that molecular diagnosis from blood specimens giving from women suspected of acute infection with *T. gondii* more specific and sensitive the serological assay (*T. gondii* specific IgM and IgG ELISA test), found about (29%) of the suspected specimens with DNA of *Toxoplasma* was identified in compared to (20%) positive bioassay, Similar results have been conducted by (Montoya *et al.*, 2002) that reported the negative serological test for women with low-avidity to Abs and negative IgM were sure negative for *T. gondii* DNA by PCR technique.

Higher significance molecular test of DNA detection in peripheral blood of aborted women infected with *T. gondii* may be due to the only technique for both the diagnose and noting of the genotyping and molecular identification clinical specimens for administered anti parasitic drugs (Costa *et al*., 2000 ; Botterel *et al*., 2002; Edvinsson *et al*., 2008 and Daval *et al*., 2010).

Conclusions and Recommendations

Conclusions:

1- Most of examined recurrent aborted women have chronic toxoplasmosis infection where IgG was positive in most cases.

2- In ABO Blood group, (B type) was common among recurrent aborted women with toxoplasma infection.

3- *B1* gene is used for detection of *T. gondii* isolated from recurrent aborted women in the present study.

4- nested polymerase chain reaction technique is the best method than others technique used in diagnosis of *T. gondii* by using tissue sample and specific primer for this parasite.

Recommendations:

1-We recommended to repeat the current study using immunohistochemistry and minividase (lgG , lgM).

2.Further studies would be to investigate other specific genes using more recent and advance diagnosis technique such as real time-PCR and microsatellite for identification bradyzoite stage of *T. gondii* in tissue samples.

3- Study the detection of *B1* gene depend on different strains of *T. gondii* to identify the role of virulence strains in seropositivily.

4-study of recent biomarkers such as (GRA6,SAG3,RE) in detection of *T. gondii* in recurrent aborted women.

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الدراسة الحالية هي واحدة من دراسات المقارنة والتي اجريت في محافظة النجف– أخذت 5 مل عينة دم من 70 نساء متكررات الاجهاض مشكوك بإصابتهن بداء القطط والتي تم فحصهن في مستشفى الزهراء التعليمي للولادة والاطفال للفترة من شهر كانون الاول 2017 الى شهر اذار 2018 ومقارنته مع 20 نساء سليمات كمجموعة سيطرة , تراوحت اعمارهم من 16- 42 سنة. 3 مل من عينة الدم تم حصول منها على مصل لتحديد الاصابة باستخدام فحص لاتكس وتقنية الامدصاص المناعي المرتبط بالأنزيم (ELISA lgG, lgM). تم اخذ 2 مل من عينة الدم وعينة النسيج لتحديد *B1* جين باستخدام تقنية تفاعل سلسلة البلمرة المتداخله (nPCR) .أظهرت النتائج نسبة عالية من داء القطط بين نساء متكررات الاجهاض.

بينت النتائج وجود ايجابية عالية بفحص اللاتكس وتقنية الامدصاص المناعي المرتبط بالأنزيم 44 (62.9%) و 33(47.1%) من اصل 70 عينة بالتعاقب. وتم تأكيد النتائج باستخدام تقنية تفاعل سلسلة البلمرة المتداخلة (nPCR) حيث كانت الايجابية للتحري عن B1 جين 12(% 17.1) و24(34.3%) من اصل70 من عينة الدم و عينة النسيج بالتعاقب. لم تحدد اي حالة أصابه بداء المقوسات الكونديه *T.gondii* ضد المستضد lgM.

علاوة على ذلك , اكدت الدراسة الحالية ان تفاعل سلسلة البلمرة المتداخله (nPCR) اكثر حساسية (100%) وخصوصية (66%) من التقنيات المستخدمة الاخرى وبمستوى عالي من الاهمية حيث كانت (*P* VALUE =0.02)

استنتجت هذه الدراسة ان تقنية تفاعل سلسلة البلمرة المتداخلة (nPCR) يعتبر من افضل التقنيات اللازمة للتشخيص داء القطط بمرحلة مبكرة من خلال التحري عن *B1* جين في عينة النسيج.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة القادسية/كلية الطب
فرع الاحياء المجهرية

دراسة الإصابة بـ*المقوسة الكوندية* أعتمادا على التوصيف الجزيئي والمناعي في النساء متكررات الأجهاض

رساله مقدمه الى
مجلس كلية الطب في جامعة القادسية
وهي جزء من متطلبات نيل درجة الماجستير في

الاحياء المجهرية الطبية

فلاح حسن هادي

بكالوريوس تقنيات تحليلات مرضية /جامعة الفرات الاوسط التقنية/

كلية التقنية الطبية والصحية/بغداد/2013

بأشراف

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