Seroprevalence and Molecular Diagnosis of *Toxoplasma gondii* in Aborted Women, Ewes and Does in Erbil Province

A Thesis
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By

Hamad Mustafa Saleh
B.Sc. Biology- Salahaddin University – Erbil– 2012

Supervised by
Assist. Prof. Dr. Zuber Ismael Hassan

Erbil–Kurdistan

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بسْمِ اللَّهِ الرَّحْمَـٰنِ الرَّحِيمِ

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Signature:

Student Name: **Hamad Mustafa Saleh**

Date: / 6 /2023

SUPERVISOR CERTIFICATE

This thesis has been written under my supervision and has been submitted for the award of the degree of Master of Medical Laboratory Technology Department with my approval as supervisor.

Signature:
Name: Assist. Prof. Dr. Zuber I. Hassan
Date: / 6 / 2023

I confirm that all requirements have been fulfilled.

Signature:
Name: Assist. Prof. Dr. Najat J. Barwary
Head of the Medical Laboratory Technology Department
Date: / 6 / 2023

I confirm that all requirements have been fulfilled.

Postgraduate Office
Signature:
Name: Zhwan M. Ismail
Date: / 6 / 2023

Examining Committee Certification

We certify that we have read this thesis: **Seroprevalence and Molecular Diagnosis of *Toxoplasma gondii* in Aborted Women, Ewes and Does in Erbil Province** and as an examining committee examined the student (**Hamad Mustafa Saleh**) in its content and what related to it. We approve that it meets the standards of a thesis for the degree of MSc. in Medical Laboratory Technology Department.

Signature
Name: **Assist. Prof. Dr. Hawri M. Bakr**
Chairman
Date: / 6 / 2023

Signature
Name: **Assist. Prof. Dr. Kareem Kh. Hamad**
Member
Date: / 6 / 2023

Signature
Name: **Assist. Prof. Dr. Abdullah A. Hama**
Member
Date: / 6 / 2023

Signature
Name: **Assist. Prof. Dr. Zuber I. Hassan**
Member and supervisor
Date: / 6 / 2023

Signature
Name: **Prof. Dr. Jawdat J. Khatab**
Dean of the College.
Date: / 6 / 2023

Dedication

- My mother and father source of love and kindness.
- My wife, Hero A Mawlud who was a beside me all the times.
- My sisters and brothers.
- My lovely sons, Mohammad and Zaid.
- All who taught me, my college, teachers and friends.

Hamad Mustafa Saleh

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ABSTRACT

The current serologic and molecular research work was designated to reassess endemicity of *Toxoplasma (T.) gondii which* is reckoned a ubiquitous zoonotic protozoan among aborted women, ewes, and does in Erbil, the Kurdistan region of Iraq. To meet the requirements of the survey, 80 aborted women, who attended both Maternity Teaching Hospitals in Erbil and Soran, were examined serologically and molecularly from November, 2021 to Abril, 2022. Moreover, 55 aborted ewes, and 30 aborted does were chosen in afore-mentioned cities at the same period and tested serologically and molecularly as well. The seroprevalence of the parasite demonstrated that 18/80 (22.5%) of aborted women had anti-toxoplasma IgG and the rest were negative. On the other hand, 4/80 (5%) women were harbors of anti-toxoplasma IgM and the rest were negative. Furthermore, the seroprevalence of the parasite demonstrated that 13/55 (23.63%) of aborted ewes had anti-toxoplasma IgG and the rest were negative. On the other hand, 2/55 (3.63%) ewes were harbors of anti-toxoplasma IgM and the rest were negative. Despite that, the seroprevalence of the parasite demonstrated that 8/30 (26.66%) of aborted does had anti-toxoplasma IgG and the rest were negative. On the other hand, 1/30 (3.33%) does were harbors of anti-toxoplasma IgM and the rest were negative. The occurrence of amplification of fragment was 100% of the toxoplasma samples. As well as, the expected patterns were provided in the samples with *T. gondii*. The result showed that the *Toxoplasma* species (Women 1, Ewes 2, and Doe 1) was 100% and ewes 12, does 7 were 99.9% homologous to *T. gondii* under the accession number (KX270387 and MK704513) due to nucleotide substitution (A → G) at the position of 207.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
PCR	Polymerase Chain Reaction
ELISA	Enzyme Linked Immunosorbent Assay
DNA	Deoxynucleic Acid
CFT	Complement Fixation Test
IgG	Immunoglobulin Gama
IgM	Immunoglobulin Mu
MAT	Modified Agglutination Test
DAT	Direct Agglutination Test
SFDT	Sabin-Feldman Dye Test
IHT	Indirect Hemagglutination Test
WB	Western Blot
LAT	Latex Agglutination Test
IFAT	Indirect Fluorescent Antibody Test
IDT	Intradermal Test
IHA	Indirect Agglutination Assay
DT	Dye Test
CNS	Central Nerve System
CSF	Cerebral Spinal Fluid
HIV	Human Immunodeficiency Virus
Ipcr	Immune Polymerase Chain Reaction
IFA	Indirect Fluorescent-Antibody
PV	Parasitophorous Vacuole
ICAM-1	Intercellular Adhesion Molecular
MIC-2	Microneme Protein
CT	Computerized Tomography
MRI	Magnatic Resonance Image
US	Ultrasound
ICT	Immunochromatograph Test
ITS-1	Internal Transcription Spacer
HRM	High-Resolution Melting
MS	Microstellite
MLST	Multi-Locus Sequencing Typing
EDTA	Ethyl Diamine Tetra Acidic Acid

BLAST	Basic Local Alignment Search Tool
µl	Micro-litter
DCs	Dendritic Cells
NK	Natural Cells
IFN	Interferon
NCBI	National Center for Biotechnology Information
MEGA	Molecular evolutionary genetic analysis
CD4	Cluster Differentiation 4
CD8	Cluster Differentiation 8
RT-PCR	Real Time Polymerase Chain Reaction
APCs	Antigen Presenting Cells
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
AIDS	Acquire Immuno Deficiency Syndrome
Th-1	T helper 1 cell
Th-2	T helper 2 cell
TNF	Tumor Necrosis Factor
IL-2,4,5,10,12,13	Interleukin-2,4,5,10,12,13
MHC	Major Histocompatibility Complex
HLA	Human Leukocyte Antigen
KO	Knockout
IgA	Immunoglobulin Alpha
IgE	Immunoglobulin Epsilon
ESA	Secretory Antigen
CLIA	Chemiluminescent immunoassay
ELFA	Electrochemiluminescent immunoassay
SNP	Single Nucleotide Polymorphism
RPM	Round Per Mniute
ECL	Electrochemiluminescence
SAG1	Surface Antigen 1
GRA	Protein secreted
ELFA	Enzyme Linked Fluorescence Assay
PCR-RFLP	Polymerase Chain Reaction -Restriction Fragment Length Polymorphism
ECLIA	Electrical Chemiluminescence Immunoassay

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

Toxoplasmosis is a cosmopolitan zoonotic parasitic disease caused by a protozoan called *Toxoplasma gondii*, the parasite is an obligate intracellular and cyst-forming protozoan capable of infecting warm-blooded animals, including humans (Ahmed et al., 2021). Humans are infected by ingestion of sporulated oocysts in water, food and soil contaminated by cat feces , or *Toxoplasma* cysts bradyzoite present in raw or undercooked meat (Pinto-Ferreira et al., 2019). The possibility of acquiring an infection displays a geographical variation and is it strongly associated with factors such as the climatic conditions, release and distribution of oocysts, the animal reservoir, consumption of meat and other personal habits (Abdelbaset et al., 2020). Risk of transmission increases with the pregnancy age, while severity of the disease for the fetus decreases. In fact, placenta barrier is more efficient at the first trimester of gestation, allowing the passage of parasites in less than 10% of infected pregnant women (Mandelbrot, 2020, Bollani et al., 2022), However, it becomes more permeable during pregnancy progress, leading to parasite transmission in 30% and to 70% of infected pregnant women in the second and third trimester respectively. Besides, infections during pregnancy can cause congenital toxoplasmosis in the fetus, resulting in spontaneous abortion, stillbirth, hydrocephalus, microcephaly, and neurological symptoms that can be detected in the uterus or at birth (Saki et al., 2021, Verteramo et al., 2022). Toxoplasmosis is an economically indispensable disease of farm animals, especially ewes and does (Abdelbaset et al., 2020). Abortion and neonatal mortality are the most important economic losses of ewes and does industries worldwide (Farag et al., 2021). In many cases of acquired toxoplasmosis, there are no symptoms or minor symptoms (Blaizot et al., 2020). The clinical symptoms differs depending on the stage of gestation, the period after infections, and the genotypes (Bonifácio et al., 2022) IgG antibodies (unlike IgM antibodies) against *T. gondii* persist for years, and can

act as a reliable serological biomarker for the diagnosis of previous exposure to this parasite (Mardani-Kataki et al., 2022). Serologic tests and PCR are used in an attempt to diagnose toxoplasmosis in pregnant women (Al-Yami et al., 2021) The diagnosis of congenital toxoplasmosis in the uterus or after birth is essential in preventing and reducing severe complications in the fetus or newborn and improving the prognosis of infection (Khan and Khan, 2018, Saki et al., 2021). Generally, maternal serologic examinations for toxoplasmosis is crucial, especially in seroconverting mothers during pregnancy, to prevent fatal injuries through medical treatment or prophylaxis (Picone et al., 2020). To identify infections during pregnancy more accurately, DNA-based molecular methods, which have higher sensitivity and specificity than serological methods, have been utilized in recent years, and different targets of *T. gondii* genome have been investigated. PCR method, using the 200-300-fold repetitive 529 bp element, is a useful diagnostic target with high sensitivity and specificity for detection of the *T. gondii* infection (Galli et al., 2019, Molaei et al., 2023). Moreover, the *B1 gene*, with the appropriate rate of variability, is considered a suitable target for molecular characterization of *T. gondii* (Arefkhah et al., 2020b). The study aim is to detect of *T. gondii* infections in aborted women, ewes, and does by serologic and molecular techniques with phylogenic tree in Erbil province.

CHAPTER TWO
LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 History of Toxoplasmosis

It has been 100 years since *T. gondii* was initially described in the tissues of *Ctenodactylus gundi*, a North African rodent, by Nicolle and Manceaux (1908) (Rouatbi et al., 2019). In the same year Splendore (1908), in Brazil, reported on the identification of this organism in the tissues of a rabbit (Vieira et al., 2018, Almeria et al., 2021). The genus was named by Nicolle and Manceaux as *Toxoplasma* for its bow-like shape (from Greek: toxo= bow or arc; plasma=creature) (Esteves, 2019). Castellani (1914**)** was probably the first to describe a *T. gondii*–like parasite in smears of the blood and spleen from a 14 year old boy from Ceylon who died from a disease characterized by severe anemia, fever and splenomegaly (Costa, 2019). Fedorovitch (1916) observed organisms similar to those reported by Castellani in the blood of a 10 year old boy from a region of the Black Sea who also had anemia, fever and splenomegaly (Oliveira, 2016). In addition, a case reported by de Lange (1929), who found protozoa in sections of the brain of a 4 month old child born with hydrocephalus, was also reviewed and re-examined by Wolf and Cowen (Elliott, 2016). Three pathologists, Wolf, Cowen, and Paige from New York, USA first conclusively identified *T. gondii* in an infant girl who was delivered full term by Caesarean section in 1938 at Babies Hospital, New York (Dubey, 2020). Following delivery, at 3 days of age this child developed seizures and chorioretinitis in both eyes. When the child died at 1 month of age, an autopsy was performed, which demonstrated free and intracellular *T. gondii* in lesions of encephalomyelitis and retinitis. Samples of cerebral cortex and spinal cord were homogenized in saline and inoculated intracerebrally into rabbits and mice; and these animals developed encephalitis from which *T. gondii* was isolated. *T. gondii* isolated from these animals was successfully passage into other mice ((Dubey, 2020). Other

forms of *Toxoplasma* including tissue cysts were recognized by several researchers including Frenkel and Friedlander (1951) (Dubey, 2020).. Burg et al. (1989) first reported detection of *T. gondii* DNA from a single tachyzoite by amplification of the *B1 gene* in a PCR (Coupe et al., 2019). Several subsequent PCR tests have been developed using different gene targets. Overall, this technique has proven very useful in the diagnosis of clinical toxoplasmosis (Döşkaya et al., 2019, Xue et al., 2021).

2.2 Classification of the Parasite

Toxoplasma gondii is a coccidian parasite with cats as the definitive host, and warm-blooded animals as intermediate hosts (Ullah et al., 2020). It is one of the most important parasites of animals. It belongs to:

Phylum: Apicomplexa
Class: Sporozoasida
Subclass: Coccidiasina
Order: Eimeriorina
Family: Toxoplasmatidae
Subfamily: Cycllosporinae
Genus: *Toxoplasma*
Species: *gondii*

2.1 Life Cycle of *T. gondii*

T. gondii is a heteroxenous parasite (Sokol-Borrelli et al., 2020). The life cycle of *T. gondii* is complex and includes two hosts: the intermediate host (such as mammals and birds), where asexual stages occur, and the definitive host (cats), where the sexual stage occurs. The *T. gondii*-genome is a haploid, except during sexual division in cats (Behnke et al., 2020).

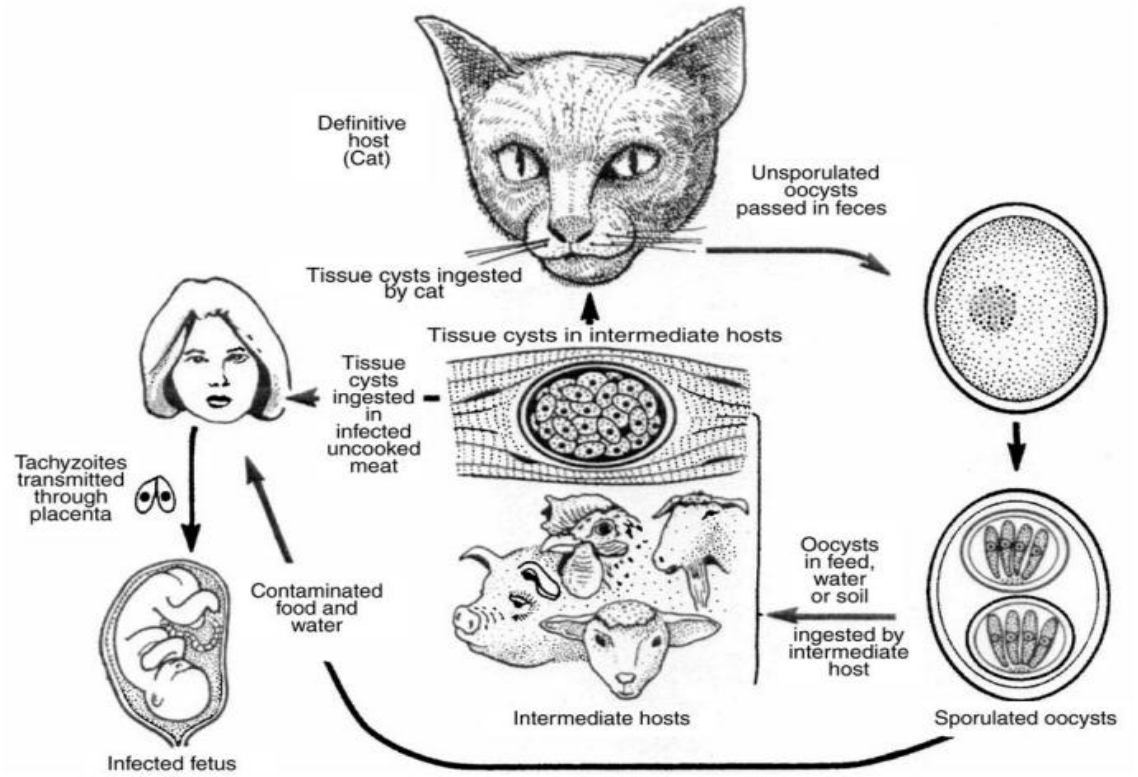

Figure (2.1) Life cycle of *Toxoplasma gondii* (Jones and Dubey, 2007)

2.3.1 Sexual Cycle

Felids ingest the parasite by preying on infected intermediate hosts including birds and rodents that contain encysted bradyzoites. Bradyzoites are released from cysts under the action of intestinal enzymes and acid digestion, and invade the epithelial cells of the small intestine. Although the parasite may disseminate throughout the definitive host's body and give rise to clinical signs, it is rarely the case (Attias et al., 2020). More frequently, in the intestine and within the course of a few days, bradyzoites will develop into different morphological enteroepithelial stages (or schizonts) to finally reach the merozoite stage (Dubey, 2016). In turn, after a few rounds of asexual division merozoites will differentiate into male (micro-) and female (macro-)

gametes. Male and female gametes will then fuse to produce diploid oocysts, which will be encapsulated in a thick impermeable wall. Millions of these will be shed in the feces of the felid and contaminate the environment. Oocysts undergo a sporulation process involving meiosis and mitosis to generate mature and infectious haploid sporozoites within now so-called sporulated oocysts (Bangoura and Daugschies, 2018). Oocysts are resistant and can persist in the environment for a long period of time, which allow their dissemination in the terrestrial or aquatic environments (Shapiro et al., 2019a).

2.3.2 Asexual Cycle

Intermediate hosts ingest sporulated oocysts through contaminated food or water. Sporozoites will invade host cells and occupy a transient parasitophorous vacuole (PV) in which they quickly differentiate into the tachyzoite form (Triana et al., 2018). Tachyzoites are highly proliferative and invasive forms that will disseminate in the host, and they are responsible for the symptoms of acute toxoplasmosis. They can travel through blood vessels or the lymphatic system and reach a number of different locations, like visceral organs, muscle, and nervous tissue. The hijacking of host immune cells allows parasite dissemination through the body (Barrett et al., 2019), and this way (but also using paracellular entry and transcellular migration) (Jeffers et al., 2018). They can also cross non-permissive biological barriers, like the blood–brain barrier, to reach immune privileged organs like the brain. In fact, immunocompetent individuals will eventually control this acute phase of infection, but coincident to the emergence of the host immune response, fast-replicating tachyzoites will differentiate into slow-growing encysted forms called bradyzoites that will remain largely hidden from the immune system (Sanchez and Besteiro, 2021). These persistent forms reside primarily in the central nervous system and muscle (Mendez and Koshy, 2017). where

they may remain for a very long time (Mendez and Koshy, 2017). This ensures parasite transmission to the definitive host to complete the cycle, at least when felids can prey on the intermediate host especially rodents. When intermediate hosts are not typical preys of felids, the parasites can still be transmitted to other intermediate hosts by carnivorism, maintaining a parasite transmission cycle without need of sexual replication (Sanchez and Besteiro, 2021).

2.4 Zoonotic Aspect of Toxoplasmosis

Toxoplasmosis is a common zoonotic disease caused by the *T. gondii* parasite, which is widespread worldwide. The disease is endemic in most areas of the world and infection can cause death in humans and animals. *T. gondii* is an intestinal coccidian of cats as the only definitive hosts and infects them by eating infected rodents, birds, or other small animals (Hill and Dubey 2018b). Cats contaminate water, soil, fodder, and vegetables by releasing millions of oocysts. Although cats are an important part of the *T. gondii* life cycle, they rarely cause clinical disease and become immune to the disease through exposure to the parasite. Other warm-blooded vertebrates including humans and most species of domestic animals and birds are considered to be the intermediate hosts. The disease can also be passed on from cats to humans by eating sporulated oocysts (Wilson et al., 2020, Sanchez and Besteiro, 2021). In the case of humans, vertical transmission from mother to the fetus, consumption of water or food contaminated with oocytes excreted by cats, and eating tissue cysts (bradyzoite) in raw or undercooked meat are the main ways of transmission of *T. gondii* to humans. In immunocompetent people, infection is clinically asymptomatic. The primary infection has subclinical features, but in some patients, ocular complications or cervical lymphadenopathy can occur. Infection during pregnancy may cause severe damages to the fetus such as abortions, stillbirths, and neonatal deaths;

moreover the infection with this protozoan causes in pregnant mental retardation, blindness, and other congenital health problems in human infants (Nayeri et al., 2021). In small ruminants especially sheep, it can lead to stillbirth, abortion, and reduced fertility (Gojam and Tulu, 2020).

2.5 Description of the causative agent like Morphology of *Toxoplasma gondii*

Infectious stages of *T. gondii*:embrace tachyzoites, bradyzoites (in tissue cysts), and sporozoites (in oocysts) (Álvarez García et al., 2021).

2.5.1 Tachyzoites

The tachyzoite is often crescent-shaped and is approximately the size (2 × 6 µm) of a red blood cell (Fig. 2.2). The anterior end of the tachyzoite is pointed, and the posterior end is round. It has a pellicle (outer covering), several organelles including subpellicular microtubules, mitochondrium, smooth and rough endoplasmic reticulum, a Golgi apparatus, apicoplast, ribosomes, a micropore, and a well-defined nucleus. The nucleus is usually situated toward the central area of the cell (Hill and Dubey, 2018a). The tachyzoite enters the host cell by active penetration of the host cell membrane and can tilt, extend, and retract as it searches for a host cell. After entering the host cell, the tachyzoite becomes ovoid in shape and is surrounded by a parasitophorous vacuole (PV). *T. gondii* in a parasitophorous vacuole is protected from host defense mechanisms. The tachyzoite multiplies asexually within the host cell by repeated divisions in which two progeny form within the parent parasite, consuming it. Tachyzoites continue to divide until the host cell is filled with parasites. Cells rupture, and free tachyzoites infect neighboring cells and the cycle is repeated. After an unknown number of cycles, *T. gondii* forms tissue cysts. (Ferguson, 2022).

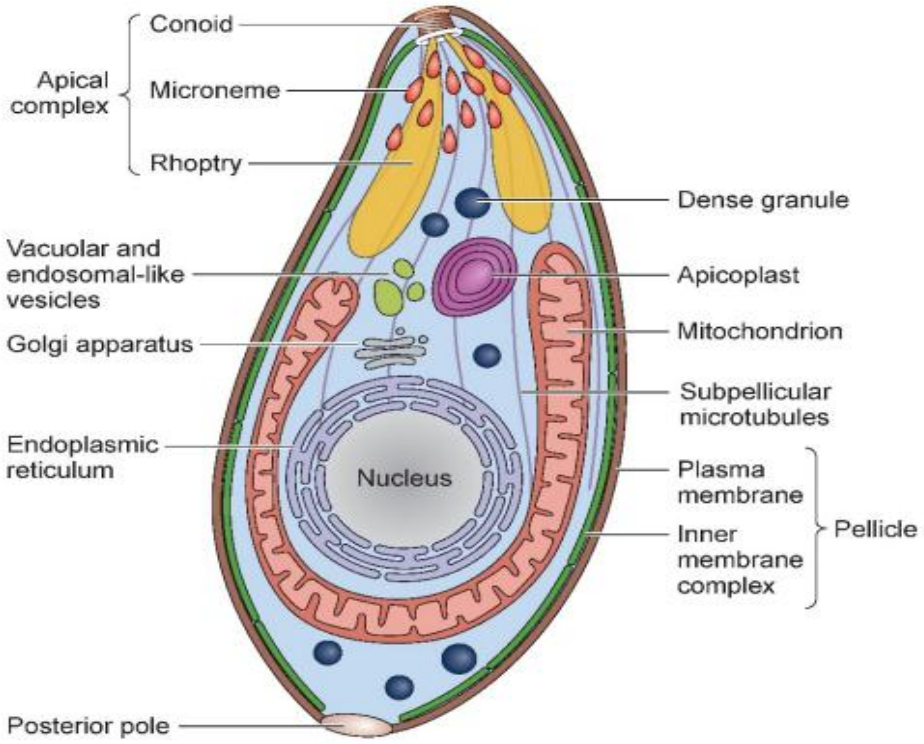

Figure (2.2): Tachyzoite from *T. gondii*, in its microscopic structure. As shown on the tachyzoites in this schematic representation, Similar to other *T. gondii* zoite forms, (Sanchez and Besteiro, 2021).

2.5.2 Bradyzoites

Tissue cysts vary in size from 5 to 70 µm and remain intracellular (Fig. 2.3). The tissue cyst wall is elastic, thin (< 0.5 µm) and may enclose hundreds of the crescent-shaped, slender *T. gondii* stage known as bradyzoites (Hill and Dubey, 2018a, Said, 2020, Elkerdany et al., 2020, Bouchard et al., 2022). The bradyzoites are approximaly (7 × 1.5 µm). Bradyzoites differ structurally only slightly from tachyzoites. They have a nucleus situated toward the posterior end whereas the nucleus in tachyzoites is more centrally located. Bradyzoites are more slender than are tachyzoites and they are less susceptible to destruction by proteolytic enzymes than are tachyzoites. Although tissue cysts containing bradyzoites may develop in visceral organs, including lungs, liver,

and kidneys, they are more prevalent in muscular and neural tissues, eye, skeletal, and cardiac muscle. Intact tissue cysts probably do not cause any harm and can persist for the life of the host (Hill and Dubey, 2018b).

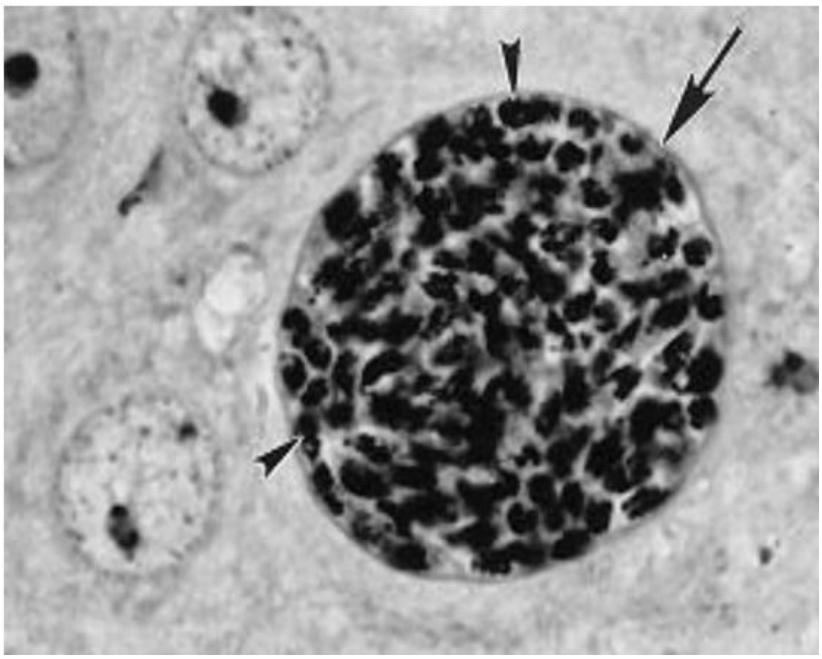

Figure (2.3): *Toxoplasma gondii* tissue cysts with abundance of bradyzoites (Jones and Dubey, 2007)

2.5.3 Oocysts

All coccidian parasites have an environmentally resistant stage in their life cycle, called the oocyst. Oocysts of *T. gondii* are formed only in cats, probably in all members of the *Felidae* (Fig. 2.4). Cats shed oocysts after ingesting any of the three infectious stages of *T. gondii*. tachyzoites, bradyzoites, and sporozoites (Jacob, 2020, Farhat, 2020). Prepatent periods (time to the shedding of oocysts after initial infection) and frequency of oocyst shedding vary according to the stage of *T. gondii* ingested. Prepatent periods are 3–10 days after ingesting tissue cysts and 18 days or more after ingesting tachyzoites or oocysts (Hill and Dubey, 2018b, Delgado et al., 2022).

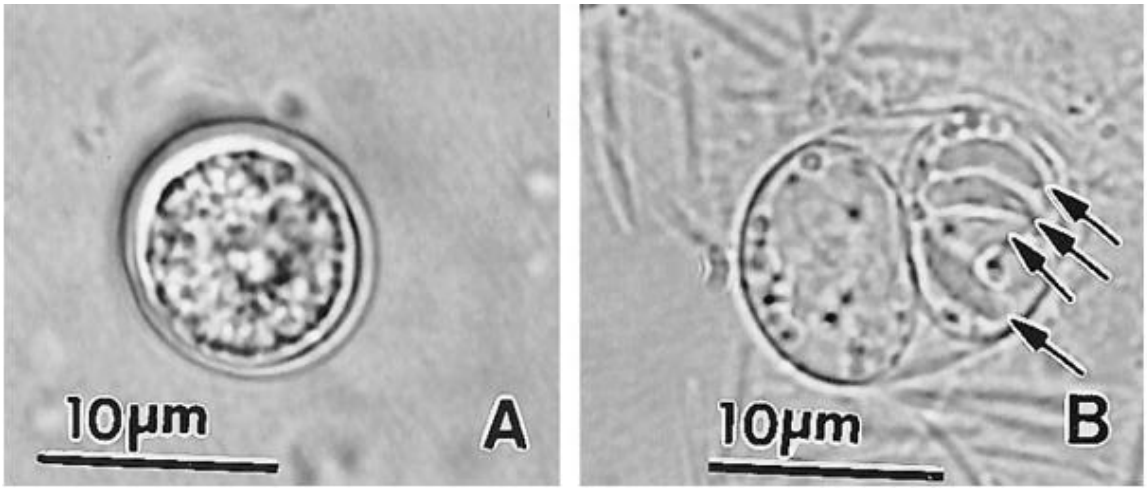

Figure (2.4): *Toxoplasma gondii* oocysts. (A) An oocyst without spores. (B) Sporulated oocyst including 2 sporocyts and containing four sporozoites in 1 of the sporocysts (Jones and Dubey, 2007)

2.6 Epidemiology and Transmission

As one of the most common parasitic infections in humans worldwide, *T. gondii* can be found in low- and middle-income countries as well as in high-income countries. In Iraq (Wasit Province) the prevalence of toxoplasmosis in women is (17%; n = 89)(Al-Sray et al., 2019) and in Baghdad is (13.6%; n = 110)(Al-Rawazq, 2017). In Australia, the prevalence of latent toxoplasmosis in women of childbearing age is 23% (n = 308) (Borkens and Wildlife, 2021). This prevalence is lower than the prevalence in other high-income countries like France (54%; n = 13459), Germany (63%; n = 4854) or New Zealand (35%; n = 500) (Borkens and Wildlife, 2021). In the United States, the prevalence in women of childbearing age is lower at only 9% (n = NA) (Borkens and Wildlife, 2021). In total, 14% of people aged 12 to 49 show a positive seroprevalence in the US (Bodenham et al., 2020). Examples for developing countries with a very high seroprevalence in women are Madagascar (84%; n = 599), Nigeria (78%; n = 352) and Cameroon (77%; n =

1014) (Ibrahim et al., 2021). While, in Kurdistan (duhok city), the prevalence of toxoplasmosis in ewes (28.9%; n = 700)(Mikaeel and Al-Saeed, 2020). In Iran, the prevalence of toxoplasmosis in ewes and does is (27.6%; n = 402) (Havakhah et al., 2014). In Turkey (13.6%; n = 1236) (Zhou et al., 2016). In Iraq (36.7%; n = 745) (HASSAN and GAEIB) Although it appears that the prevalence of *T. gondii* is not influenced by the climate (Waap et al., 2022). warm climates and low-lying areas seems to favour the infection (Borkens and Wildlife, 2021). Human can get toxoplasmosis by two ways

2.6.1 Horizontal Transmission

Epidemiologically several ways conduce to infection *T. gondii*. Infection via the faecal-oral route by ingestion of contaminated water or undercooked vegetables with sporulated oocytes is common (Polley et al., 2022). On the other hand the meat of infected animals contains cysts of bradyzoites is another causes of infection(Opsteegh et al., 2020). Humans can become infected after consumption of undercooked meat (especially pork, lamb and venison; transmission through beef is very rare) (Gaulin et al., 2020). Infection can also occur after the consumption of animal milk containing tachyzoites of *T. gondii* (Almeria and Dubey, 2021). Infections via blood donations or organ transplants are also possible (Stopić et al., 2022).

2.6.2 Vertical Transmission

This happens when the mother becomes infected during pregnancy (Buonsenso et al., 2022). Vertical transmission can also occur if the toxoplasmosis of a chronically infected woman is reactivated in the course of an immunosuppressive disease, or if seroconversion occurs in an immunocompetent woman only a few months before conception (Gómez-Chávez et al., 2020). Vertical transmission can be asymptomatic (Ahmed et

al., 2020). As the definitive host of *T. gondii*, cats play an important role in the lifecycle of *T. gondii* as well as its maintenance in nature (Gering et al., 2021). Infections in cats occur horizontally by ingestion of tissue of intermediate hosts as well as vertically (Pal et al., 2021). Inside the cat intestine, *T. gondii* undergoes sexual reproduction that results in the production of oocysts. The quantity of oocyst produced varies between 3 and 810 million per cat infection (Coupe, 2021). The oocysts will be shed with faeces in the environment; there they can survive from several months up to one year (Shapiro et al., 2019a). Thus, cats are able to spread many oocysts widely, which in turn increases the likelihood of infecting intermediate hosts. Chronically infected cats, however, show a certain resistance due to *T. gondii* antibodies and shed oocysts more seldomly (Borkens and Wildlife, 2021). There are several risk factors for domestic cats to contract *T. gondii*. Cats fed with fresh or wet food show a higher seroprevalence than cats fed with dry food (Davies et al., 2019, Wingertzahn and Sharma, 2022). A further risk factor are cats, both domestic or feral, who roam in the environment. These cats are more likely to become infected with *T. gondii* as a result of praying on small mammals, scavenging on carcasses and ingestion of oocysts(Coupe, 2021). To reduce that risk, pet cats should be deterred from hunting (Udonsom et al., 2021). Cat owners are at risk from close contact with cats and their faecal material (Li et al., 2022).

2.7 Pathogenesis

The placenta prevents the passage of infectious agents towards the foetal compartment more effectively at the beginning of pregnancy than at the end. It is a key tissue in the mother-to-foetus relationship, not only because of its trophic role but also because it provides the tolerant immune microenvironment necessary for gestation (El-Sherbini et al., 2019). During primary infection, parasites cross the intestinal barrier and invade monocytes

in contact with the lamina propria, which allow them to disseminate through the blood flow towards virtually all organs, including placenta (Seo et al., 2020). Infection of the placental tissue can result in a placentitis and can lead to subsequent infection of trophoblast cells, which are at the interface with the foetal compartment and may let the parasites proceed (Teixeira et al., 2021). The majority of lesions in the placental tissue are necrotic, both the number as well as size of lesions intense on gestation progresses. Early in the process of pregnancy, an infection results in necrotic lesions that affect the caruncular septa also an elevation in the numbers of inflammatory cells, primarily macrophages as well as lymphocytes, in the embryonic villi nearby the lesions. Infection that occurs later in the course of pregnancy causes more severe harm to the placental tissue, which develops more numerous and broad necrotic foci that affect simultaneously maternal and fetal organs. *T.* gondii can invade and multiply within trophoblast cells (Almeida et al., 2021), but the mechanisms by which this otherwise effective barrier can fail to protect the foetus, allowing some pathogens to enter, remain unclear although some hypotheses have been suggested. Whereas an efficient immune response against *Toxoplasma* requires a T helper Th-1 cytokine pathway response involving interferon g (IFN-g) (Yap et al., 2000, Clark et al., 2021, Sang et al., 2022). The placental microenvironment is rich in interleukin 10 (IL-10) and promotes a Th-2 immune response to ensure maternal–foetal tolerance (Pastor-Fernández et al., 2021) which could facilitate infection of placental tissue, The interplay between immune effectors of successful pregnancy and of anti-infectious response has been extensively described elsewher (Pfaff et al., 2007, Barbosa et al., 2008, Milian et al., 2019, Vueba et al., 2020). The pivotal cytokine in this complex process is IFN-g, as shown in a mouse model where IFN-g synthesis following *T. gondii* infection led to abortion in pregnant wild type mice, but not in pregnant IFN-g knockout (KO) mice (Sireci et al., 2021). Such a deleterious effect of IFN-g is also described in

preeclampsia in humans (Hackmon et al., 2008, Dijkstra et al., 2022). Thus, a delicate balance exists between the anti-*T. gondii* effector functions of IFN-g and its abortogenic effects, and both maternal and foetal environments contribute to this complex equilibrium. However, the role of IFN-g could be more ambiguous, as it was shown in vitro that it upregulates the expression of intercellular adhesion molecule (ICAM)-1 adhesin at the trophoblast cell surface and thereby contributes to enhanced adhesion of infected monocyte (Brenier-Pinchart et al., 2011, Teixeira et al., 2021). In addition, ICAM-1 is induced during placentitis (Fakonti et al., 2021). and could directly support transepithelial migration of the parasites, as it was shown to coprecipitate with the microneme protein MIC-2 (Brenier-Pinchart et al., 2011, Kim et al., 2022). MIC-2 is secreted by the parasite and relocalised at the parasite surface during cell invasion (Mahmoudzadeh et al., 2021). Local mechanisms could compensate for infection induced deleterious effects. Placental cells have the unique capacity to select specific genes within the major histocompatibility complex (MHC); amongst which is human leukocyte antigen (HLA)-G that may be responsible for the reprogramming of local maternal immune response to facilitate maternal–foetal tolerance (Tantengco et al., 2021). During *Toxoplasma* infection, HLA-G could contribute to counterbalance the cytotoxic Th-1 immune response (Fernández-Delgado et al., 2020, Ikumi and Matjila, 2022). In a recent study, higher levels of soluble HLA-G were observed in amniotic fluid from foetus, whose mothers had a history of *Toxoplasma* infection during pregnancy than in those without infection (Robert-Gangneux et al., 2011, Tantengco et al., 2021). Multiple foci of necrosis are caused by the placenta's parasites multiplying, and abnormalities in the placenta might result in abortion (Piao et al., 2018). Recent diverse hypotheses, which allowed progress in the understanding of trophoblast cell infection and immune regulation at the maternal–foetal interface are described.

2.8 Diagnosis

Toxoplasmosis can be diagnosed using a number of different methods. Due to the absence of specific as well as distinct clinical toxoplasmosis symptoms, there are numerous techniques used to detects *T. gondii* infections, which are divided into serological as well as non-serological assays (Abdelbaset et al., 2020).

2.8.1 Serological Tests

Several serological methods, including the modifed agglutination test (MAT), direct agglutination test (DAT), Sabin–Feldman dye test (DT), indirect immunofuorescence test (IFAT), Western blot (WB), latex agglutination test (LAT), indirect hemagglutination assay (IHA), and enzyme-linked immunosorbent assay (ELISA) have been applied to identify *T. gondii*-specifc antibodies in both humans and animals (Damour, 2018, Sigle et al., 2020).. The main technique for diagnosis of *Toxoplasma* infection is to detect anti-*Toxoplasma*-specific antibodies (Molaei et al., 2022). The principle of all serological test is based on exposing suspensions of parasite antigen to the sera of suspected sample to see whether they contain antibodies or not by observing occurrence of reaction between both. Diagnosis had been done classically by monitoring a particular immunoglobulin M (IgM) antibody or by specific immunoglobulin G (IgG) antibody or both, other reports have emphasized the value of detecting specific IgA or IgE antibodies of *T. gondii* for diagnosis of early phase of toxoplasmosis (Vueba, 2022). The IgM antibody typically can be detected within days of infection while IgG antibodies can be detected within 1-2 weeks and may persist for life. The existence of IgM antibodies suggests an acute infection (Osoro et al., 2022, Status—United).

The following serological tests are in use for diagnosis of *T. gondii* infection:

2.8.1.1 Immunochromatograph Test (ICT)

The immunochromatographic test is a rapid detection technique in which the colloidal gold-labeled antigen or antibody is used as the tracer, and the cellulose membrane is used as the solid support (Fig. 2.5) (Wang et al., 2022) and the detection antibodies or antigens are dropped at the sample pad on the nitrocellulose membrane, which will slowly infiltrate the conjugated pad through capillary action, and antibody-antigen complexes show colloidal gold color reaction (Doménech-Carbó and Doménech-Carbó, 2022). A rapid immunochromatographic strip using colloid gold conjugated antiexcretory/secretory antigens (ESA) IgG antibodies was developed to detect ESA in acute infection of *T. gondii* as early as 2–4 days post-infection, showing high agreement with ELISA in sensitivity and specificity (Rostami et al., 2018). The antibody detection results of GRA7-, SAG2- based ICT are consistent with those of LAT and ELISA. As ICT is easy, rapid, and convenient to perform, and no special equipment is required, it is suitable for field application.

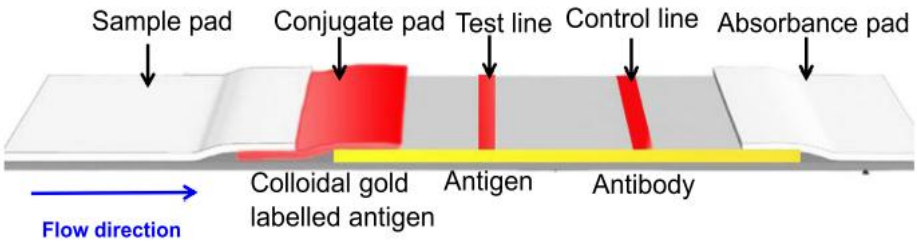

Figure (2.5): Flowchart showing the immunochromatographic analysis to identify antibodies against *T. gondii*. Antigen either antibody coated with colloidal gold is utilized as a tracer, and cellulose membranes is utilized as a solid support (Liu et al., 2015a)

2.8.1.2 Automation Testing Techniques

Involve many methods such as, chemiluminescent immunoassay (CLIA), and electrochemiluminescent immunoassay (ECLIA). Because of its high sensitivity and specificity, its used for routine screening work. These methods recognize *Toxoplasma* infection either by detection of IgG antibodies specific for the parasite, or detection of IgM antibodies which is helpful to differentiate between past and recent infection, use of IgG avidity test in case that IgM remain for several months for determination the time of infection (Souza et al., 2012).

2.8.1.2.1 Cobas e 411

Cobas e 411 machine is based on Electrochemiluminescence technique have the ability to detect anti-toxoplasma IgM antibodies during the early stage of acute infection better than ELISA and ELFA technique, and confirmed the usefulness to diagnose acute toxoplasmosis during the first trimester of pregnancy (Meylan et al., 2015).

2.8.2 Molecular Techniques Depending on Parasitic Nucleic Acid Detection

Molecular methods are used in addition to conventional serological methods for the diagnosis of toxoplasmosis. Conventional methods are usually not misleading, but are limited in prenatal cases or in immunocompromised patients (Abdelbaset et al., 2020). For example, a mother may be diagnosed accurately by serology that she has had a current infection during pregnancy and so her baby is potentially at risk of congenital infection but the serology results cannot confirm whether the parasite has been transferred to the baby. However, the molecular diagnostic techniques may do so.

2.8.2.1 Conventional Polymerase Chain Reaction (PCR)

Due to inherent limitations of traditional diagnostic methods, PCR can be used in addition to serology to diagnose *T. gondii* infection. PCR is an efficient in vitro enzymatic amplification method that allows specific amplification of DNA from minute amounts of starting material in a short time (Biswas and Nandi, 2018). To achieve high sensitivity, several multicopy targeting genes are usually used for the detection of *T. gondii* in biological samples, including the *B1 gene*, the 529 bp repeat element and the internal transcribed spacer (ITS-1) or 18S rDNA sequences. The presence of a parasitaemia is seldom detected therefore PCR of blood has a low negative predictive value. Several other single-copy genes, such as SAG1, SAG2, and GRA1, have also been used as PCR targets in some laboratories (Zrelli et al., 2022). This method has widely been used in prenatal diagnosis of congenital toxoplasmosis and *T. gondii* infection in immunocompromised patients (Khan and Khan, 2018, Singh et al., 2022). repeat element was reported to be 10- to 100-times more sensitive than the *B1 gene* (Dai et al., 2019). The multicopy ITS-1 and 18S rDNA have also been used as the targets in a few studies, showing a similar sensitivity of the *B1 gene* (Veronesi et al., 2017). To further improve the sensitivity and specificity, nested PCRs based on the *B1 gene*, the 529 bp repeat element, and ITS-1 sequences have been developed (Chemoh et al., 2016). In the nested PCR, two sets of primers are used in two successive PCRs. The products of the first reaction are used as templates for the second PCR. For a given targeting gene, nested PCR is more sensitive than the conventional PCR. The detection limit of the 529 bp repeat element-nested PCR is 640 fragment of parasite DNA, while the rate for B1-nested PCR is 5.12 pg (Liu et al., 2015b), and the nested PCR targeting the *B1 gene* is more sensitive than targeting ITS-1 sequence (Liu et al., 2015b). The sequence of the PCR product must be verified to provide adequate diagnostic specificity.

2.9 Genotyping Techniques According to Molecular Technology

For epidemiological studies, it is important to identify genotypes of *T. gondii* infection, and some molecular technologies, including microsatellite analysis, multi-locus sequence typing, PCR-RFLP, RAPD-PCR, and high-resolution melting (HRM) analysis, have been developed.

2.9.1 Micro-satellite Analyses

Microsatellite (MS) sequences are tandem short DNA motif repeats that are widespread in eukaryotic genomes and the sequences usually change due to insertion or deletion of repeat units. The numbers of repeat units differ in a population, thus producing multiple alleles at an MS locus. The tandem repeats in *T. gondii* are often simple, and composed with as few as 2 nucleotides, and occur 2–20 times. (Liu et al., 2015b, Anghel et al., 2021). A total of 15 MS markers, including TUB2, W35, TgM-A, B18, B17; M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, and N83, have been used to genotype *T. gondii* in different laboratories (Santos et al., 2018, Deiró et al., 2021). developed an easy-to-use method for genotyping *T. gondii* in a single multiplex PCR assay using 15 microsatellite markers, in which the 8 MS markers (TUB2, W35, TgM-A, B18, B17, M33, IV.1, and XI.1) could differentiate types I, II, and III from all the atypical genotypes, and the other 7 markers (M48, M102, N60, N82, AA, N61, and N83) could enhance genetic resolution in differentiating closely related isolates within one haplogroup or clonal lineage (Deiró et al., 2021). The 15-MS multiplex assay is the best tool available to identify *T. gondii* isolates genetically different or identical, to identify the infection source in an outbreak, laboratory contamination and mixed infections (Shapiro et al., 2019b). The limitation of this assay is the requirement for an automated sequencer. In addition, small amount of DNA

from biological samples could cause the absence of detectable peaks or peaks of low intensity, which is undistinguishable from nonspecific PCR products (Liu et al., 2015b). The microsatellite analysis, are grouped into one of three clonal lineages of type I, II or III (Deiró et al., 2021). These strains have been described in Portugal, with types II and III as common in pigs and chicken (Abdelbaset et al., 2020) . Studies in other European countries, including France, Spain, Switzerland, and Germany, have revealed a predominance of genotype II in domestic animals , (Fernández-Escobar et al., 2020).

2.9.2 Multiple Locus Sequencing Typing (MLST)

Multilocus sequence typing (MLST) is based on DNA sequence polymorphisms, including the single nucleotide polymorphisms (SNPs), and deletion and insertion of nucleotides, which has the highest resolution among all typing methods when enough genomic DNA is available (Bekő et al., 2019). Several studies have revealed some alleles unique to the Brazil isolates, including 5′-SAG2, 3′-SAG2, GRA6, and SAG3 (Pena et al., 2021). However, this approach is not a good choice for clinical samples, as a large quantity of genomic DNA is required for this assay.

2.10 Response of the Immunity System to *Toxoplasma* Infections

Both innate immunity, which include the initial protection, and adaptive immunity, which is divided in to cellular and humoral immunity, are involved in the immunological response to toxoplasmosis (Ivanova et al., 2019).

2.10.1 Innate Immunity Response to *Toxoplasma gondii*

The main components involved in the innate immune response are macrophage, dendritic cells (DCs), natural killer (NK) cells and cytokines (Negishi et al., 2018). The innate immune response performs a key function to limit the infection in the event of a microbial invasion, until adaptive immunity is introduced. Tachyzoites induce macrophage to generate cytokine interleukin in the acute phase of infection. The development of IL-12 is an important element of the immuno-cascade during *Toxoplasma* infection, to produce protective IFN and to eliminate virulent tachyzoites (Mahmoudzadeh et al., 2021). Interleukin-12 acts on NK cells that initiate interferon-gamma (IFNγ) production (Hamid et al., 2021). In the acute and chronic stages of *T. gondii* infection, IFNβ stimulates the killing of phagocytosed tachyzoites and it is considered a significant mediator on host resistance (Hou et al., 2022). During *Toxoplasma* infection, the NK cell contribute to early IFNα production. It is thought that CD4 and CD8 T cells are the primary sources of this cytokine (Ribot et al., 2021). Beside to IL-12, Myloid cells produce TNF to stimulate production of additional IFNγ by lymphocyte and works as a co-factor to activate macrophage's microbicide mechanisms (Denkers and Microbiology, 2003). In fact, cytotoxicity and cytokine formation are NK cell functions (Poznanski and Ashkar, 2019). Pro-inflammatory cytokines from DCs, neutrophils and macrophages stimulate IFNγ production by NK cells, which mediates early parasite resistance in the early stage of *Toxoplasma* infection. NK cells increase cytotoxicity in *Toxoplasma*-infected mice and target both extracellular and intracellular parasites (Briukhovetska et al., 2020).

2.10.2 Adaptive Immunity Response

Humoral and cellular immunity that including antibodies and T-cells (CD4+ and CD8+ T lymphocytes) essential in providing immunity to infection with *T. gondii* (Abdelbaset et al., 2020, Zhu et al., 2020).

2.10.2.1 Cell Mediated Immunity Responses

The cell-mediated immunity is a fundamental component of host immune reactions to *Toxoplasma gondii* infection (Gao et al., 2018). The cell-mediated response in a host protects against acute *T. gondii* infection and also controls the chronic stage (Khan et al., 2019). Long-term protection will mainly be mediated by CD8 T cells and CD4 Th-1 cells by IFN γ and IL-2 production (Otero, 2019). The IFN γ, TNFβ and IL-2 are produced by Th1 cells and IL-4, IL-5, IL-10 and IL-13 are secrete by the Th-2 cells (Sen et al., 2021). Toxoplasmosis immunity is correlated with a Th-1 response type (Mercer et al., 2020). Synergistic impacts of CD4+ and CD8+ T cells and their relative importance were proved via depletion of one or both. The decline of CD4+T cells in naïve mouse increased susceptibility and cyst burden and death following inoculation with a virulent *Toxoplasma gondii* strain (Suzuki, 2020). The depletion of either CD4+ or CD8+ T cells in chronic infected mouse did not trigger reactivation, while depletion of both subsets, which led to the activation of infection (Ren et al., 2020). The *T. gondii* peptide antigens are displayed in the APCs via their MHC class II molecules (Hammed-Akanmu et al., 2022) When the peptide antigens of *T. gondii* are recognized by Naïve CD4 T-lymphocytes, these cells begin to differentiate, and undergo clonal expansion. After secretion of IL-12 by macrophage and other APCs, the cytokine acts on the naïve CD4 cells which leads to formation of Th-1 effector cell subset (Groft et al., 2020). The Th-1

cells secrete a set of cytokines that cause inflammatory reaction and stimulate the formation of anti-*Toxoplasma* antibodies. On the other hand, Naïve CD8 T cells, recognize MHC class I bound *T. gondii* antigens, and in combination with IL-12 induced co-stimulatory signals they are activated and differentiated into CD8 T lymphocyte with cytolytic activity (Women, 2015). The affected cells with tachyzoites are recognized and destroyed by cytolytic CD8 lymphocytes and infection reservoirs are destroyed during the acute phase (Smith et al., 2021).

2.10.2.2 Humoral Immunity Response

The production of high concentrations of circulating specific antibodies is also followed by the initiation of a powerful cellular immune response. If the B cell reaction occurs, affinity maturation and isotype switching occur and a IgG level (high avidity) is detectable in the serum. The roles of antibodies in *T. gondii* resistance have not yet been clarified (Swale et al., 2022). The antibodies can destroy the parasites either by activating the complement system, which results in parasite lysis or by opposing the parasite to phagocytic cells for phagocytosis (Ramesh, 2018). The antibodies concentrations drop after the immune response controls the parasite but remain high throughout the host's lives. Presence of cysts and rarely discharging of tachyzoites may lead to chronical stimulation of immunity of the hosts and a maintenance of measurable concentrations of antibodies that manifest the latent phase of parasite infection. Therefore, the presence of antibodies (rising IgG and the presence of IgM antibodies specific for *T. gondii*) determines the infected persons with *T. gondii (Holec-Gąsior and Sołowińska, 2022)*. Immunoglobulin G antibodies generally appear 1 to 2 weeks after the infection it reaches to a maximum level within 1 to 2 months then decreased at different rates and generally lasts for the whole life. On the

other hand, IgM antibodies may appear sooner than IgG antibodies and decrease more quickly (Abdelbaset et al., 2020).

CHAPTER THREE

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Materials

The laboratory instruments, equipment and chemical materials, as well as kits and materials of molecular characterization which used in this study are illustrated in tables (3.1), (3.2) and (3.3) respectively.

Table (3.1): instruments and equipments used throughout the study

No	Instrument and Equipment	Supplies	Origin
1	EDTA Tube	Biozek	Netherlands
2	Gel Tube	Biozek	Netherlands
3	Centrifuge (Micro and Head)	Mikro Hettich	Taiwan
4	Refrigerator, temperature range +1 - +8°C	LG	Korea
5	Deep freeze -20 to -80 ^{o}C is used for long term storage of samples	LG	Korea
7	MiniVortex	Isolab	UK
8	Beaker, Conical Flask, Cylinder	Kern	China
9	Autoclave	Wisd	USA
10	Balance	Kern	China
11	Incubator	Memmert	China
13	Micropipettes difference in size	Sterilin	UK
14	Eppendorf Tube (1.5, 2.0ml)	Eppendorf	Germany
15	Pipette Tip	Eppendorf	Germany
16	Ice crash	Jouan	China
17	Nano drop	Thermo	USA
18	Biohazard hood	Jouan	China
19	Sterilized cotton swabs	Falcon	USA
20	Urine container	Biotech	Turkey
21	Rack	Alpha	India
22	Water bath	GFL	China
23	Ice back	Kern	China
24	Timer	Kern	China
25	Ethanol 70%, 96%	Alpha	India
26	Para film	Par film M	USA

Table (3.2): Device and kits used throughout the study

No	Device and kits	Supplier	Origin
1	Cobas e 411	Hitachi	Korea
2	Toxoplasma Kit	Elecsys	Germany
3	Toxoplasma strip	Biozek	Netherlands

Table (3.3): Commercial kits and materials used for the molecular study

No	Molecular kit and materials	Supplier	Origin
1	PCR Device	Bioer XP	China
2	PCR tube	Alpha	India
3	Gel electrophoresis device	Consort	China
4	DNA extraction kit	Bio-Tech	Korea
5	Primers (Forward, Reverse)	Macrogen	Korea
7	PCR water	Macrogen	Korea
8	Master mix	Ampliqon	Denmark
9	Safe gel stain	Addbio	Korea
10	DNA ladder(50 and 100 bp)	GeneDirex	USA
11	Agarose	Geneon	Germany
12	TBE buffer	Cleaver scientific	UK
13	UV trans-illuminator	Biotec Fischer	Germany
15	Microwave	LG	Korea
16	PCR rack	Macrogen	Korea

3.2 Methods

3.2.1 The Study Area and Sample Collection

This cross-sectional study was carried out in Erbil province from November 2021 to April 2022. Samples blood and placental tissues were collected from 80 aborted women aged 16 to 48 years old that had a spontaneous abortion or were having pregnancy problems at Erbil Maternity Teaching Hospital and Soran Maternity Teaching Hospital, in additions 85 aborted animals (55 ewes and 30 does). The blood was separated and kept at -20 °C at refrigerator until use for immunological assay. Furthermore,

placental tissue biopsies were taken from aborted women, ewes and does throughout the first, second and third trimesters and stored in 70% of ethanol. The whole blood and placental tissue were kept for DNA extraction.

3.3 Blood preparation

Five ml of venous blood sample was withdrawn from antecubital fossa or median cephalic vein of each aborted woman, ewes and does using disposable syringes after cleaning the area for 30 seconds with an alcohol wipe. Two ml of blood was placed in EDTA tube for genomic extraction, the remaining three ml of blood was placed into un activated tube (gel tube) and kept at room temperature for clotting, and then the serum was separated by centrifuge at 3000 round per minute (rpm) for about 10 minutes. Each serum sample was analyzed for the presence of *T. gondii* by identifying serum IgG and IgM antibodies using initial test (immunochromatograph) and Cobas e-411 analyzer.

3.4 Placental Preparation

A fragment of placenta tissue about 15–30 gm was removed and cut into small pieces, then placed in a 10 ml of collection tube.

1- Deionised water was added to the tubes.
2- The liquid phase was removed by aspiration, preventing the removing of the germinal layers, and the solution was re-suspended by vortexing.
3- To completely eliminate of ethanol from placenta tissue, processes 2 and 3 were repeated three times.

3.5 Serological preparation

3.5.1 Analytical Technique

Depending on the manufacturer (based Elecsys test Kit Germany), the process for detecting anti-toxoplasma IgM and IgG was under:

1. Wash solution system was prepared by adding a 30 ml of wash system to 3 ml of deionized water.

2. The pro cell and the clean cell were placed in their special places in the machine.
3. Both assay cup and tips were put in their places in the machine.
4. Then the (start) bottom was pressed.
5. The user name and password were entered.
6. Maintenance process and system reset were performed.
7. The kits were placed in the reagent disk.
8. Scan reagents were performed
9. The calibrator was placed in the sample disk (the capacity 30 test)
10. The barcode special for each test was read through (barcode scan).
11. The calibration processes were performed.
12. Reagent sample was added to control and it has been read by a special barcode using a (control barcode). The control process was conducted and the results were within the SD1 level and close to the mean which indicates the accuracy of the results according to the levelling chart.
13. Tests were conducted on the collected samples.

3.5.2 The principle assays guiding

1. First incubation: involve dilution of sample (10) µL in a ratio (1: 20) automatically with Universal Diluents. After that added *T. gondii*-Ag which is labelled with a ruthenium complex. Followed by reaction of (IgM) antibodies with the ruthenium-labelled *T. gondii*.
2. Second incubation: included adding both Biotinylated monoclonal h-IgM-particular antibodies with streptavidin-covered microparticles to form complex. Then this complex binds to the solid phase through reaction between streptavidin and biotin
3. Inside the measuring cell, aspiration of the reaction mixture occurred where the microparticles were attracted magnetically on top of electrode surface; then using ProCell/ProCell M to eliminate unbound

substances. Use of passing voltage through the electrode followed by chemiluminescent emission that measured by using a photomultiplier.

4. The software responsible for determination of the results automatically by comparing between the emission signals of electrochemiluminescence and value of the cut off that determine by calibration (Meylan et al., 2015).

3.6 Serology Test for Toxoplasmosis

Cobas e 411-based Elecsys was done using kits (Elecsys) laboratories to recognize IgG and IgM-specific immunoglobulin anti *T. gondii* antigens in the patient's serum.

3.6.1 Immunochromatograph test (ICT)

The immunochromatographic test is a rapid detection technique in which the colloidal gold-labelled antigen or antibody is used as the trace, and the cellulose membrane is used as the solid support (Ibrahim, 2020) and the detection antibodies or antigens are dropped at the sample pad on the nitrocellulose membrane, which will slowly infiltrate the conjugated pad through capillary action, and antibody-antigen complexes show colloidal gold colour reaction (Ibrahim, 2020) . A rapid immunochromatographic strip using colloid gold conjugated antiexcretory/secretory antigens (ESA) IgG antibodies was developed to detect ESA in acute infection of *T. gondii* as early as 2–4 days' post-infection. The antibody detection results of GRA7-, SAG2- based ICT are consistent with those of LAT and ELISA. As ICT is easy, rapid, and convenient to perform, and no special equipment is required, it is suitable for field application (Ibrahim, 2020).

Figure (3.1) Immunochromatograph test

3.6.2 Identification of (IgM and IgG) specific anti-toxoplasma gondii immunoglobulin via utilizing Electrochemiluminescence (ECL) methods

Kits for detection of IgM and IgG antibodies against *T. gondii* antigens in human sera were used according to the company`s information. Anti-toxoplasma IgM and IgG detection were used to evaluate a patient's serologic status to *T. gondii* infection. The sera were examined for anti-Toxoplasma IgM and IgG antibodies using an automated analyser platform (Cobas e 411 analyser) which is a fully automated machine and immunoassay analysis controlled by software system. The software performed automatically transmission of information to and from the analyser, results assessment, documentation and quality control. Cobas e 411 is based on electrochemilumenisce (ECL) technique which is "Electro" refers to electrical stimulation, "Chem" point to a chemical reaction "Luminescence" refers to light production. This technology provides superior analytical performance and increased sensitivity which means that it can detect extremely low level of antigen, as well as slight changes in antigen level (Souza et al., 2012).

Figure (3.2): Cobas e 411 analyser

3.7 Primers for *Toxoplasma*

The *B1 gene* was employed for identification of *T. gondii* isolates by PCR (Maani et al., 2021). The B1 primers used were: forward (5'CGCTGCAGGGAGGAAGACGAAAGTTG3') and that of reverse was (5'CGCTGCAGACACAGTGCATCTGGATT3').

3.7.1 Primer preparation (stock and working solutions)

3.7.2 Procedure for Stock solution

1. The packets was opened, the primer tube was removed and cleaned with tissue papers.

2. The tube was spied down in a micro centrifuge, at 3000 rpm for a few seconds before opening, so that none of the primer falls out.

3. To make a 100 µM solution, 10 times the amount deionized distilled water was

(100µM Master Stock) = x nmoles of lyophilized primer*10µl of grade water.

4- Vortexed for 30-60 seconds to be mixed well.

5-Spined down in the micro centrifuge again and stored in a deep freezer at -20^{0}C.

3.7.3 Working solution

1- To generate 10 μM working dilution of the primer, the stock solution was diluted by the following formula:

$$(\text{Volume}_{\text{initial}} * \text{concentration}_{\text{initial}} = \text{Volume}_{\text{final}} * \text{concentration}_{\text{final}})$$

2- Stored in a deep freezer at -20^0C.

3.7.4 Proteinase K

Proteinase K is prepared commonly as a 20 mg/ml stock solution in 2.5 ml sterile water (stable for one year at -20^o).

3.8 Genomic DNA Extraction

DNA extraction from tissue placenta was performed used a commercial DNA extraction kit {Bio-Tech, Korea} as per the manufacturer's protocol, the extraction process was achieved in four steps: (Lysing, Binding, Washing and Elution) as follow:

1- Transfer a fragment of placenta to micro-centrifuge tube and add 200 microliter of lysis buffer.
2- 20 microliters were added of proteinase K solution to the sample tube, vortex the mixture and incubated at 56C^o. Once the tissue is totally lysed.
3- Rotated the tube down slowly to clear any droplets from the specimen tube's lid inside.
4- 200 microliters were of binding buffer to the specimen tube and mix well thoroughly by vortex for 15 seconds, then incubate at 56C^o for ten minutes.
5- 200 microliters were added of absolute 96% ethanol and mix well by vortex for 15 second.
6- Gently transfer the lysed into the upper reservoir of the spins column tube using the 2 ml collecting tube avoid wetting the rim

7- Centrifuging at 13000 rpm after one minute, pour off from the flow-through then construct the spin column using the 2.0 ml collector tube.
8- Added 500 microliters of Washing Buffer 1 to spin column collector tube and centrifuging at 13000 rpm for 1 minute, pour off from the flow-through then construct the spin column using the 2.0 ml collector tube.
9- Added 500 microliter of washing buffer 2 to spin column collector tube and centrifuging at 13000 rpm for one minute, pour off from the flow-through then construct the spin column using the 2.0 ml collector tube.
10- To eliminate any remaining ethanol in the spin columns, centrifuge the spin column once more at 13000 rpm for one minute.
11- A clean 1.5 ml micro-centrifuge tubes should be used to transfer the spin column (not provided).
12- Added 80-100 microliters of elution buffer added to it within the micro-centrifuge tubes then allow settle for at least one minute.
13- Elute the genome DNA by centrifuge at 13000 rpm for 1 minute to obtain extracted DNA.

3.9 Determination of DNA Concentration

By utilizing a nano drop spectrophotometer, the concentration and purify of the isolated DNA were determined. (Thermo Fisher Scientific, USA) purified DNA at absorbance (260/280 nm), at rate 1.8, and carried out in accordance (Bunu et al., 2020). Before being utilized for PCR amplify, DNA was kept at 20^{-} °C.

3.10 PCR Amplification and Primers

3.10.1 PCR Amplification

Due to the inherent limits of traditional diagnosis techniques, PCR can be applied to identify *T. gondii* infections. With the use of the effective in *vitro* enzymatic amplification technique known as PCR, DNA may be specifically amplified from small amounts of starting materials in a short duration of time (Yousefvand et al., 2022). The *B1 gene* is typically employed for the identification of *T. gondii* in clinical specimen in order to obtain high sensitivity. A *B1 gene* was the focus of the first *T. gondii* identification PCR technique, which was developed in 1989 (Rostami et al., 2018).

3.10.2 PCR and Gene Amplification

For gene amplification, *B1 gene* for *Toxoplasma gondii* was specified for amplifies. For this study, a conventional PCR (Gene Q Thermal Cycler, Bioer Technology) instrument was used. A specific section of the DNA template was amplified using (PCR) in order to generate sufficient copies for testing.

Figure (3.3) Thermocycler

3.10.3 Detection of *B1 Gene* of *Toxoplasma gondii* Isolates by PCR

The identification of *B1 gene* by (PCR) technique was done as described (Schares et al., 2021). the maximum volume of reactions 25 µL containing 12.5 microliter of Master Mix, 7.5 PCR water, 1.5 µL from both of them forward and reverse prime, 2 µL of the DNA extraction sample as a template, The PCR tubes were closed, combined by a vortex, then centrifuged for a brief period of time at highest speed. The necessary reagents for the PCR reactions were combined in a sterilized PCR tube, which was then placed on ice for storage at each step of working. The maximum of amplification for single sample illustrated in table (3.4). The amplifying reaction was carried out under the following condition: initially denatured (94^{o}C for 4 minute), Following, 35 cycles of denaturation (95^{o}C for 45 second), annealing (55^{o}C for 45 second), elongation (72^{o}C for 45 second), and final extension step (72^{o}C for 5 minute). The PCR conditions were as illustrated in table (3.5).

Table (3.4): Mixture of Amplification for a Single Sample: Compounds and Volumes

No.	PCR mixture name	Volume
1	Master mix	12.5 µL
2	PCR water	7.5 µL
3	Prime forward	1.5 µL
4	Prime reverse	1.5 µL
5	DNA extraction	2 µL
	Total	25 µL

Table (3.5): The Thermocycler Conditions for *B1 gene*

Step	Temperature	Time	No. of cycles
Initial denaturation	94.0 °C	4 minutes	1
Denaturation	95.0 °C	45 seconds	35
Annealing	55.0 °C	45 second	
Extension	72.0 °C	45 second	
Final extension	72.0 °C	5 minutes	1

3.11 Agarose Gel Electrophoresis for the Screening of Amplified Products

The PCR products were assessed by electrophoresis in 1.5 % agarose gel stained with safe dye. Agarose gel was prepared by dissolving 0.5 gm of agarose powder in 33 ml of Tris-acetate-EDTA Buffer (TAE) in microwave, allowed to cool to 45°C and then a safe dye at the concentration of 4 µL/100 ml was added (Zamora-Ballesteros et al., 2022). combs were fixed at one end of the tray for making wells used for loading DNA sample. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 minunte. The comb was then removed gently from the tray. The gel tray was placed horizontally in the electrophoresis tank, so that, the wells in the gel become towards the cathode, then the tank was filled with TAE 1x buffer to just cover the gel to a depth of about one cm. 5 µl of PCR product transferred to each well and 5µl of DNA ladder added to the first well (Lanphere et al., 2021). The gel chamber closed, the power source switched on. For a better resolution, power supply of 50 volts was applied for 15 minutes until the DNA left the wells and moved about (0.5) cm towards the

positive electrode. Then, the voltage was increased to 95 volts, and the electrophoresis was allowed to proceed for a sufficient time.

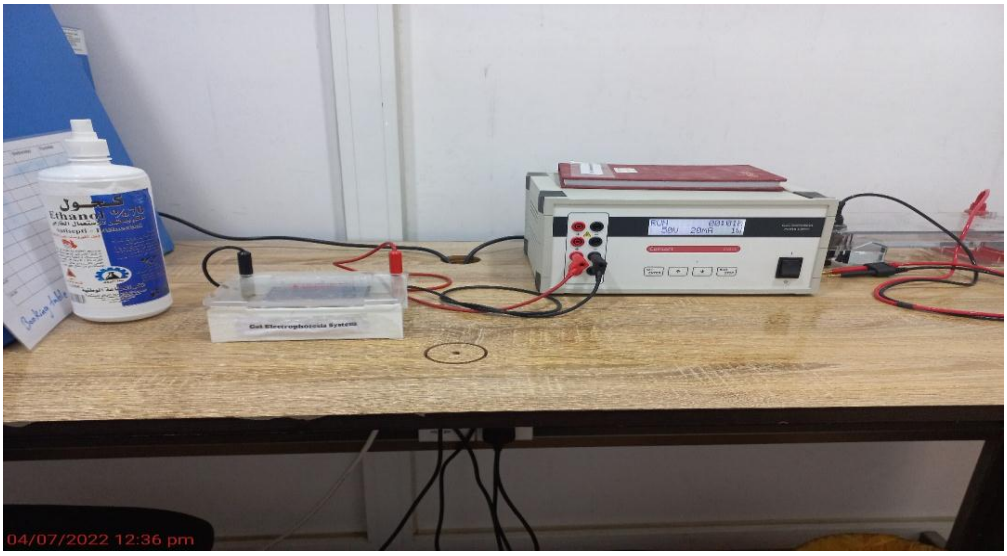

Figure (3.4) gel electrophoresis apparatus

UV Trans-illuminator was used for the observation of DNA bands, and then gel was photographed with a digital camera. 100 bp DNA ladder was used as molecular markers (Gatta and Al-Graibawi, 2021).

Figure (3.5) UV Trans-illuminator

3.12 Nucleotide Sequencing and Phylogenetic Analysis

The PCR products of *Toxoplasma* were verified by analyzing the nucleotide sequences of the *B1 gene*. The sequences were aligned through the ClustalW algorithm (Thompson et al., 1994), provided by BioEdit v7.2.5(Hall, 1999), with sequences available in the GenBank (NCBI) database. Nucleotide sequences of the partial *B1 gene* of the *T. gondii* from aborted women, ewes, and does were analyzed, and the phylogenetic tree was performed on individual partial gene sequences utilizing MEGA-6 software (Molecular Evolutionary Genetics Analysis) http://www.megasoftware and the neighbour-joining were used to build the tree.

3.13 Statistical Analysis

Scatter plot graphing software (Graph Pad Prism v.7, CA, USA) was used for data analyses. The Chi-square test was used to analyze the data, and a P value ($P<0.05$) was ordered as statistically significant.

CHAPTER FOUR

RESULT AND DISCUSSION

4. RESULT AND DISCUSSION

4.1 Seroprevalence Studies of Aborted Women

Out of 80 women, who have had spontaneous abortions, the median age 28 years (ranging from 16–48 years) in the first, second and third trimesters, were clinically diagnosed and examineted for detection of *Toxoplasma* infection by Cobas e 411. The rate of infections was 18 (22.5%) for IgG and 4 (5%) for IgM as shown in table (4.1). The result agreed with study of (Ali, 2018), which demonstrated that the prevalence of *T. gondii* seropositivity using ELISA technique, screening for IgG type antibodies specific to *T. gondi* 250 samples, there were 65 (26%) seropositive in overall groups; in normal birth group there were 14 (14%) seropositive cases out of 100-screened samples and in abortion group from 150 samples 51 (34%) were seropositive .The statistical analysis appears significant difference at $P < 0.01$,while screening for IgM type antibodies specific to *T. gondi* 250 samples, there were 50 (20%) seropositive in overall groups; in normal birth group there were 10 (10%) seropositive cases out of 100-screened samples and in abortion group from 150 samples 40 (26.6%) were seropositive .The statistical analysis appears a significant difference at $P < 0.01$. As well as, Taha et al. (2019) in Sudan who revealed that, out of the 152 blood sample collected from aborted women, 54 were found positive for *Toxoplasma* IgG with prevalence rate (35.5%), while only one IgM positive with prevalence rate (0.6%). Furthermore, Mahmood et al. (2021) in Iraq who showed that, out of 63 aborted women in Kirkuk reported that 6 (10%) were reactive with both IgG and IgM immunoglobulin, while 6 (10%) reported seropositive for only IgM immunoglobulin, and 19 (30%) reported seropositive for only IgG immunoglobulin. Furthermore, Lachkhem et al. (2020) in Tunisia who expressed that. In recent infections with *T. gondii*; particularly IgM immunoglobulin is first recognized and produced

after acute *T. gondii* infection and IgM typically turns negative after a few months (Teimouri et al., 2020, Elzeky et al., 2022). Anti-Toxoplasma IgG immunoglobulin antibodies usually appear within 1–2 weeks of acquisition of the infection, peak within 1–2 months, decline at various rates, and usually persist for life. As well as, in patients with recently acquired primary infection, *T. gondii*– specific IgM antibodies are detected initially, and in most cases, these titers become negative within a few months. However, in some patients, positive *T. gondii*–specific IgM titers can still be observed during the chronic phase of infection (Montoya, 2002).

Table (4.1): Seropositivity of *T. gondii* infections in aborted women attended Maternity Teaching Hospitals in Erbil Province using Cobas e 411

Erbil and Soran maternity teaching hospitals	Total aborted No.	Anti-toxoplasma IgG	(%)	Anti-toxoplasma IgM	(%)	Total	P value
Erbil Maternity Teaching Hospital	47	10	12.5	3	3.75	13	0.6161
Soran Maternity Teaching Hospital	33	8	10	1	1.25	9	
Total	80	18	22.5	4	5	22	

The results in (Table 4.2) revaled that the distribution of *Toxoplasma* seropositivity using cobas e 411 technique in relation to residence area. Screening for IgG type antibodies specific to *T. gondii* high seropositivity in rural area were 11 (13.75%) out of 80 samples and low seropositivity in urban area were 7 (8.75%). Furthrmore, screening for IgM type antibodies specific to *T. gondii*. There were 4 (5%) seropositive cases out of 80 screened samples, high seropositivity in rural area were 3 (3.75%) and low seropositivity in urban area were 1 (1.25%). This result is in consistence with that of (Ades et al., 1993, Ertug et al., 2005). This finding is similar to that reported in Malaysia (Altunoluk et al., 2000) and in Egypt (Attia et al., 1995); in India (Joshi et al., 1998) and in Erbil city (Joshi et al., 1998) in which they

observed that the disease is apparently more prevalent among rural aborted women than those living in urban community, but with no significant difference. The seroincidence variation between the two communities depends on the basis of poor standards of hygiene and lower socioeconomic status in rural area than in the urban, also rural women are more in contact with domestic and other animals than urban women. The pattern of risk factors and their relative importance are likely to vary appreciably across national boundaries in accordance with cultural patterns and climatic factors affecting the survival of oocysts (Ali, 2018).

Table (4.2): Seropositivity of *T. gondii* infections in aborted women according residency

Residency	Total No. (80)	Anti-toxoplasma IgG	(%)	Anti-toxoplasma IgM	(%)	positive No.	(%)	P Value
Rural	59	11	13.75	3	3.75	14	17.5	>0.9999
Urban	21	7	8.75	1	1.25	8	10	

Women with a low educational level showed more at risk of being infected with *T. gondii* (table 4.3). Patients who had completed high school were clearly shown to be protected against anti- *T. gondii* seropositivity. Several other authors who have studied toxoplasmosis among aborted women have also found similar results (Mohammed and Al-Janabi, 2019).

Table (4.3): Seropositivity of *T. gondii* infections in aborted women according educational level

Educational level	Total No.	Anti-toxoplasma IgG	(%)	Anti-toxoplasma IgM	(%)	Total positive No.	(%)	P Value
Uneducated	23	7	8.75	1	1.25	8	10	0.8416
Elementary	30	6	7.5	1	1.25	7	8.75	
Secondary school	16	3	3.75	1	1.25	4	5	
University	11	2	2.5	1	1.25	3	3.75	
Total	80	18	22.5	4	5	22	27.5	

This study was conducted on 80 aborted women, their ages ranged between 16-48 years old, the age groups were divided into < 20, 20-29, 30-39 and ≥ 40 years old. (Table 4.4) demonstrate the seroprevalence was observed higher in the age range between 20 to 29 years 8 (10%), 2 (2.5%) for Toxo-IgG and IgM respectivly. The lowest rate was found in ≥ 40 years which was 1 (1.25%) for IgG and in 30-39 years which was zero for IgM. These results were in agreement with several previous studies from different regions (Khalil, 2008, Nasser et al., 2019). that indicated the percentage of anti-*T . gondii* antibodies was a higher with the increase of age group in Iraq While the results disagrees with the studies that revealed the higher percentage of seropositive women to *T. gondii* between (21-25) years age group (Al-Dujaily, 1998).

The results also differed with other studies which observed that the main age of seropositive for toxoplasmosis cases are between (11-20) years old (Al-Rawi, 2009, Abdullah, 2016) who concluded that the main age of seropositive of toxoplasmosis cases were between (20-30) years old. The high occurrence of toxoplasmosis with this age may be due to marriages of this age group, which is considered the age of higher fertility so we found that most of pregnant women were young 16-19 years old. However ,when IgG antibodies

against *T. gondii* were present in the blood they indicate that *T.gondii* cyst were already present in the tissue (Dupon et al., 1995).

Table (4.4): Seropositivity of *T. gondii* infections in aborted women according to the age group

Age group	Total No.	Anti-toxoplasma IgG	%	Anti-toxoplasma IgM	%	Total	%	P Value
< 20 years	15	3	3.75	1	1.25	4	5	0.4167
20-29 years	31	8	10	2	2.5	10	12.5	
30-39 years	22	6	7.5	0	0	6	7.5	
≥40 years	12	1	1.25	1	1.25	2	2.5	

Table 4.5 shows the first trimester recorded higher rate of anti-toxoplasma antibodies IgG and IgM in comparison to son an second trimester and the lowest rate was found in the third trimester (p value = 0.6463). Such results were comparable to those obtained (Akoijam et al., 2002), they found that no significant association between abortion and infection with *T. gondli*. The result partialy agreed with (Ali, 2018), she concluded in her results that *T. gondii* agglutinins prevalence in women suffering from abortion in Erbil city, also the same findings were indicated on aborted women in Mosul city, Duhok and Baghdad (Jasim, 1979, Ali, 2018, Al-Khaffaf, 2001). This variance may be explained by the presence of different source for distribution of the parasite in this locality and may be due to the difference in sanitation and hygienic way of living; or the difference may be due to the influence of climate on the survival of toxoplasma oocysts in the environment, in addition different eating habits and differences in husbandry of domestic animals may affect exposure to infection (Ashburn, 1992).

Table (4.5): Seropositivity of *T. gondii* infections in aborted women according to the gestational age of abortion

Gestational age of abortion	Total No.	Anti-toxoplasma IgG	(%)	Anti-toxoplasma IgM	(%)	Total positive No.	(%)	P Value
1st trimester	51	12	15	2	2.5	14	17.5	0.6463
2nd trimester	18	5	6.25	2	2.5	7	8.75	
3rd trimester	11	1	1.25	0	0	1	1.25	

As shown in the table (4.6), the seropositivity of IgG was detected 10 (12.5%) and IgM was 3 (3.75%) of patients who had past history of abortion. The result agreed with the studies of Mohammed and Al-Janabi (2019) which showed that the frequency of previous abortion was highest (17.3%) in patients having two previous abortions. The current study did not found a significant relationship between the percentage of infection and the number of abortions (table 4.7), although it reported high rates of infection among women who experienced double abortion. The current study was compatible with the study conducted by Shaker et al. (2018) who found that the number of abortions to two times higher than the number of abortions for once.

Table (4.6): Seropositivity of *T. gondii* infections in aborted women according to history of abortion

History of abortion	Total No.	Anti-toxoplasma IgG		Anti- toxoplasma IgM		Total positive No.	(%)	P Value
		No.	(%)	No.	(%)			
Yes	53	10	12.5	3	3.75	13	16.25	0.6161
No	27	8	10	1	1.25	9	11.25	

Table (4.7): Seropositivity of *T. gondii* infections in aborted women according to frequency of abortion

Frequency of abortion	Total No.	Anti-toxoplasma IgG		Anti-toxoplasma IgM		Total positive No.	(%)	P Value
		No.	(%)	No.	(%)			
No	29	6	7.5	1	1.25	7	8.75	0.9065
Single	41	9	11.25	2	2.5	11	13.75	
2 or more than 2	10	3	3.75	1	1.25	4	5	

Women who kept cats as pets had a noticeably increased percentage of *T. gondii* seropositivity 11 (13.75%) and 3 (3.75%) for Anti-Toxo- IgG and IgM immunoglobulin show in table (4.8). The result agreed with Elsheikh (2015) in Wad madani who reported seroprevalence of 41.7% using latex. This study nearly closed to our findings, although, he studied the relation of some risk factors to the disease, and found that consuming raw meat and contact with pets other than cats and dogs were highly significant to infection.

Table (4.8): Seropositivity of *T. gondii* infections in aborted women according to presence of cats

Presence of cats	Total No.	Anti-toxoplasma IgG		Anti-toxoplasma IgM		Total positive No.	(%)	P Value
		No.	(%)	No.	(%)			
Yes	49	11	13.75	3	3.75	14	17.5	>0.9999
No	31	7	8.75	1	1.25	8	10	

4.2 Seroprevalence studies of aborted ewes and does

Serum samples were collected from 55 aborted ewes and 30 aborted does in Erbil Province (Table 4.9). Out of 55 aborted ewes investigated serologically, 15 (27.27%) in ewes were positive. Among them, 13 (23.63%) ewes were carries of anti-toxoplasma IgG, While 2 (3.63%) ewes were holders of anti-toxoplasma IgM. From 30 does examined 9 (30%) were seropositive for *T. gondii*. Aomng them 8 (26.66%) does were harbors of anti-toxoplasma IgM, while 1 (3.33%) does was carrier of anti-toxoplasma IgM.

Statistically, no significant difference (P=0.05) was observed for both ewes and does. The result of the present study showed be similar with the study in Pakistan carried out by Hanif and Tasawar (2016) who observed that the seroprevalence of toxoplasmosis was 27.4% by ELISA. Also, the results of the present showed be similar with other results as in Misan province-Iraq and Turkey, were the prevalence of toxoplasmosis were 25% and 31.4% by using ELISA respectively (Oncel and Vural, 2006, Khadi et al., 2009). While (Amairia et al., 2016) revealed that, a total number of 77 blood samples were collected from six herds were screened with a commercial ELISA kit for *T. gondii* antibodies. The seroprevalence of *T. gondii* infection was 31.2% . The variation in prevalence is due to variations in health management, feeding and watering systems, nutrition regimens for pregnant animals, biosecurity systems, geographical locations, season, weather, carrier intensification, parasitic genomic diversity, and genetic marker polymorphisms significantly influence the percentage of abortion (Arefkhah et al., 2020a, Alemayehu et al., 2021).

Table (4.9): Seropositivity of *T. gondii* infections in aborted ewes and does

Species	number of samples	No. of positive sample	(%)	Anti-toxoplasma IgG		Anti-toxoplasma IgM		P Value
				No.	(%)	No.	(%)	
Aborted ewes	55	15	27.27	13	23.63	2	3.63	>0.9999
Aborted does	30	9	30	8	26.66	1	3.33	
Total	85	24	28.23	21	24.70	3	3.52	

The seropositivity rate of IgG and IgM antibodies to *Toxoplasma* which was no statistically different ($P \geq 0.37$) between different ages groups of ewes by cobas e 411. The rate was higher in the age group more than 3 years 12 (21.81%) as compared in age with less than 3 years 3 (5.45%), as shown in Table (4.10). While in does, the rate was higher in the age group more than 3

years 5 (16.66%) as compared in age with less than 3 years 4 (13.33%). This is proved by Lashari and Tasawar (2010) in Pakistan who stated that the rate of infection was the highest (38.88%) in age group of 16-28 months and the lowest (8.51%) in age group of 68-80 months by using ELISA. Also, these results were approved by Al-Barwary and Mikail (2014) in Duhok Province that have shown the ewes with age group < 4 years 61.24% as higher than ewes with age groups ≥ 4 years 17.02% by ELISA. While it is disagreed with the results of the study done by Al-Taie and Abdulla (2011) in Sulaimania, in which sheep with 7-9 years (6/8) 75% were higher than ≤ 3 years (20/53) 37.7% by ELISA and by Subedi et al. (2018) in Nepal, who showed that the seroprevalence was higher in ewes of more than 2 years of age 82.35% while lower in age group of 2 or less than 2 years of age 17.65% by ELISA. This may be due to the fact that younger ewes have less resistance and also, they can be actively exposed to oocysts and have been having more chance to acquire infection. On the other hand, Leyva et al. (2001), it has been documented that *T. gondii* infection usually leads to long-term protective immunity.

Table (4.10): Seropositivity of *T. gondii* infections in aborted ewes and does according to ages

Species	Ages	number of samples	No. of positive sample	(%)	Anti-toxoplasma IgG		Anti-toxoplasma IgM		P Value
					No.	(%)	No.	(%)	
Ewes	Less than 3 years	22	3	5.45	2	3.63	1	1.81	0.37
	More than 3 years	33	12	21.81	11	20.00	1	1.82	
Does	Less than 3 years	9	4	13.33	4	13.33	0	0.00	>0.99
	More than 3 years	21	5	16.66	4	13.33	1	3.33	

Table (4.11) showed that there was no statistically significant difference at P ≤ 0.05 in those ewes and does that aborted once or more than one time when estimated of the antibodies in sera by cobas e 411. The rates were 11 (20%) and 4 (7.27%) respectively according to the frequency of abortion. While in does, the rates were 7 (23.33%) and 2 (6.66%) respectively. This is approved by results obtained at by Mikail (2007) in Duhok in which ewes with single abortion (136/365) 37.26% were higher than those of 2 abortion (12/39) 30.77% by using ELISA.

Table (4.11): Seropositivity of *T. gondii* infections in aborted ewes and goats according to the number of abortions

Species	Number of abortion	number of samples	No. positive sample	(%)	Anti-toxoplasma IgG		Anti-toxoplasma IgM		P Value
					No.	(%)	No.	(%)	
Ewes	One abortion	40	11	20.00	9	16.36	2	3.63	>0.99
	More than one abortion	15	4	7.27	4	7.27	0	0.00	
Does	One abortion	27	7	23.33	6	20.00	1	3.33	>0.99
	More than one abortion	3	2	6.66	2	6.66	0	0.00	

4.3 Molecular Studies

To confirm the serological results and the presence of *T. gondii* infection, PCR was done for some of seropositive samples that were randomly selected. The TOX4 and TOX5 was amplified to be used for species specific\ strains. The obtained data demonstrated that the entire placental tissue isolated from aborted women, ewes and does after amplification products were electrophoresed and visulazed using UV transluminator, which showed clear and visible bands. The size of the bands was the same in all women, ewes and does isolates which was about 529 bp on 1.5% agarose gel after staining

with sefe gel stain as shown in figures (4.1) which was the same bands generated by primer measured for DNA size marker (50 bp and100 bp DNA ladder).

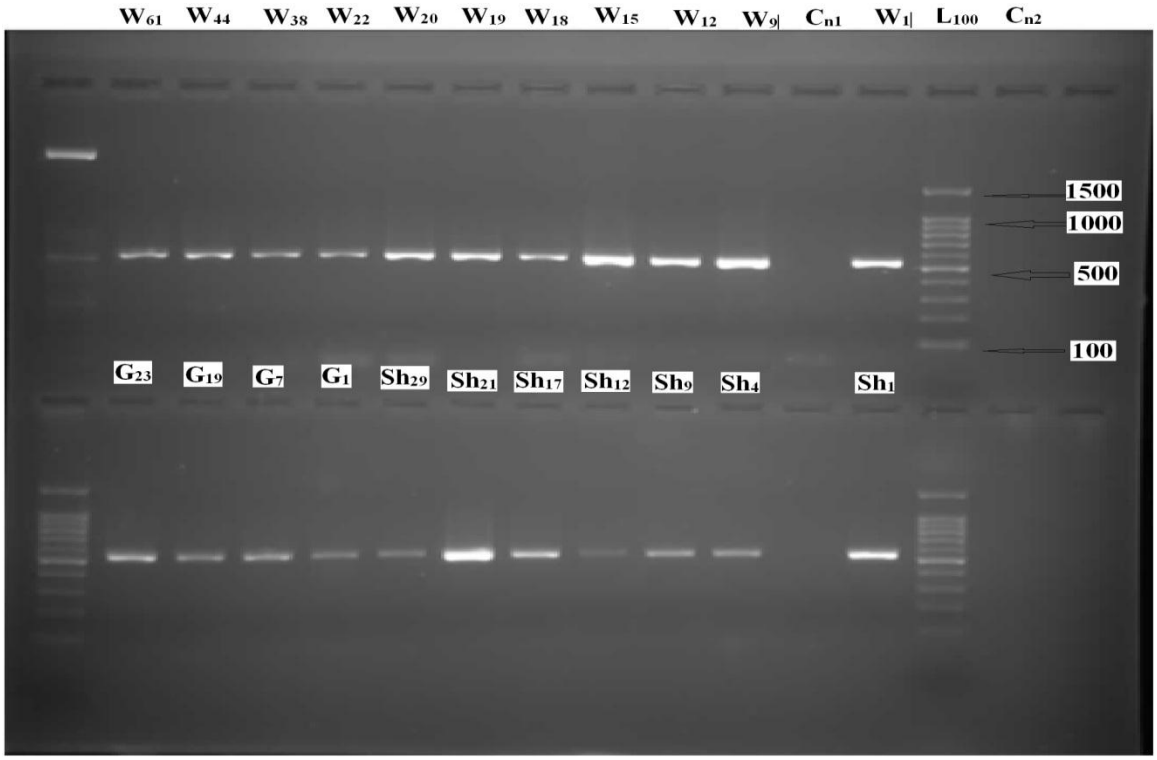

Figure (4.1): Gel electrophoresis of the PCR products. Lanes (W1, W9, W12, W15, W18, W19, W20, W22, W38, W44 and W61) denote women samples, (Sh1, Sh4, Sh9, Sh12, Sh17, Sh21 and Sh29) denote sheep and Lanes (G1, G7, G19 and G23) denotes goats samples amplified as a single band of 529 bp; C_{n1} denotes DNA extraction negative control; C_{n2} denote PCR negative control, and 100 bp denote 100 bp ladder molecular weight marker.

The placental tissue and whole blood with seropositivity by cobas e 411 (18 women, 15 ewes and 9 does) were tested for targeting the *B1 gene* utilizing conventional PCR. The results revealed that 11 women, 7 ewes and 4 does were positive and obviously showed an amplicon size of 529 bp. The result agreed with Shaapan et al. (2021), who revealed that the seropositive samples of both ewes and mares were randomly selected for PCR to validate the results of ELISA. In the mares, all samples were found to be positive by

ELISA, while in the case of ewes, only 60 samples were found positive by ELISA. These samples were randomly selected to be verified by amplification *B1 gene* using PCR. The PCR result shows two positive samples out of 11 in mares at a rate (18.2%) and 13 positive samples out of 60 were positive in ewes at the rate (21.7%), clearly showing an amplicon size of 529 bp. On the other hand, (ISSAD et al., 2022) reported that *T. gondii* DNA was diagnosed in 114 females out of 307 placenta tissue samples (37.1%) by conventional PCR based on *B1 gene* amplification. Furthermore, Hasan et al. (2021) revealed that 87 tissue samples were examined for *T. gondii*, which was found through the *B1 gene*. Seven tissue specimens were positive for *T. gondii*. Intriguingly, nested PCR results for the heart muscle, liver, brain, and placenta were reactive. In addition, lung samples from this investigation produced negative results. Positive outcomes were more prevalent in the placenta and brain than in the heart muscle. Comparing lung and liver sample results, the lung sample yielded the least adverse findings. The lack of protozoa in the blood at the moment of blood collection and the parasite's localization as tissue cysts, tachyzoites, and/or bradyzoites within the host's body are thought to be the causes of the reduced ratio by PCR compared to the cobas results. Additionally, the acute infection may have subsided or changed into a persistent infection (Lopes et al., 2015).

The occurrence of amplification of fragments was 100 % of the *Toxoplasma* samples. As well, the expected patterns were provided in the samples with *T. gondii*, as shown in figure (4.2). The result showed that the *Toxoplasma* species (Women 1, ewes 2, and does 1) was 100% and ewes 12, does 7 were 99.9% homologous to *T. gondii* under the accession number (KX270387 and MK704513) due to nucleotide substitution (A → G) at the position of 207 as shown in Figure (4.2). The nucleotide sequences agreed with the result and were consistent with *T. gondii* (Martínez-Flores et al., 2017, Shamaev et al., 2020, Mikaeel and Al-Saeed, 2020, Shaapan et al., 2021). Thus, it was found

that our isolates were near the type strain (Abdul Hafeez et al., 2022). The molecular method used for the isolation of *Toxoplasma* species resolves the time-consuming and difficulties in interpreting some morphological, and physiological patterns and confirmation of strains (Fernández-Escobar et al., 2020, Salehi et al., 2020). The different cultures and probably the ethnical, climatic and geographical elements and features of cases are responsible for factors determining this variation (Amouei et al., 2022).

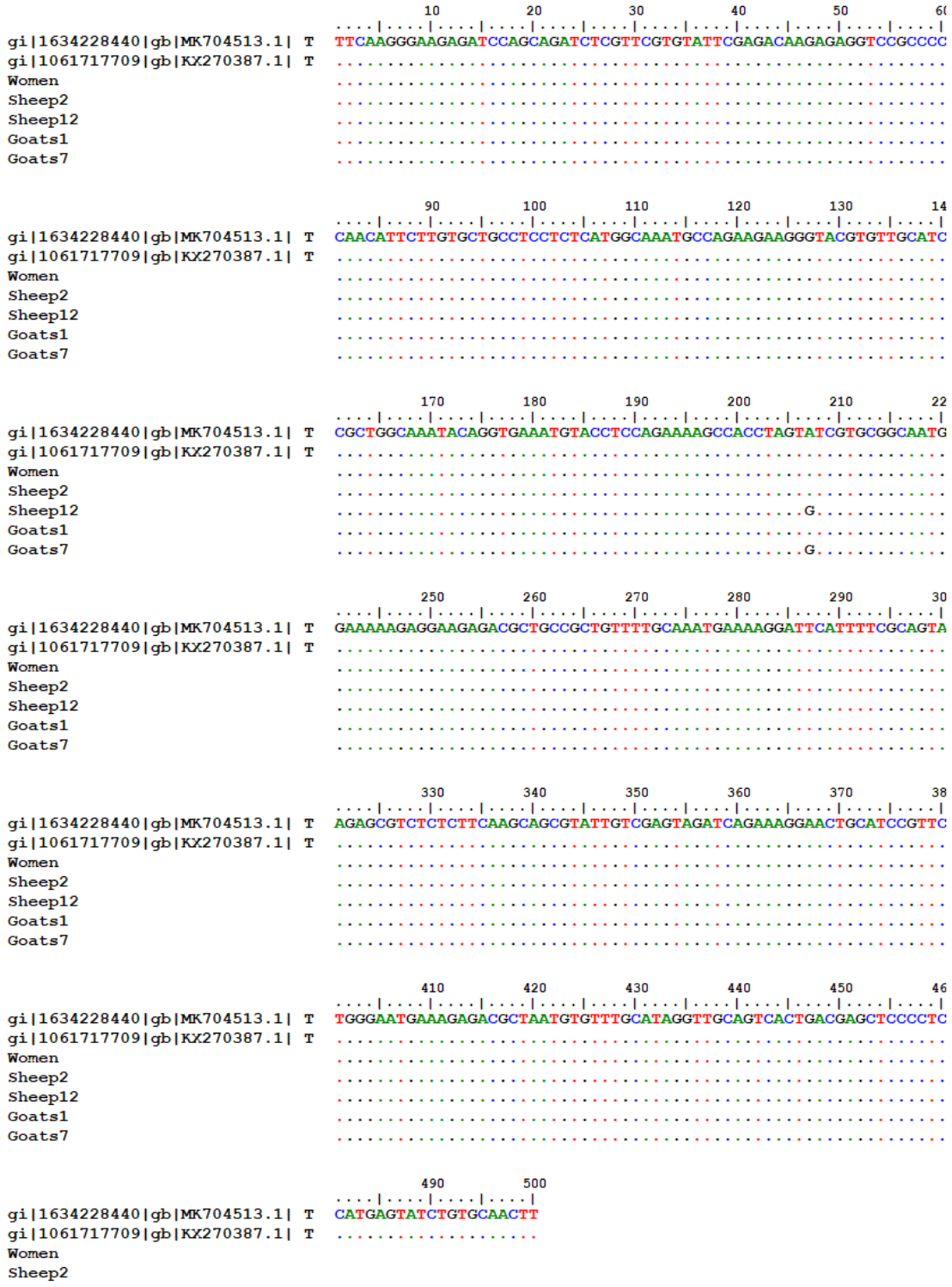

Figure (4.2): Alignment of a fragment of *T. gondii* (women, sheep 2, 12 and goats 1, 7) with GenBank accession numbers KX270387 and MK704513

Toxoplasma species (women, ewes 2,12 and does 1,7) were compared with reference sequences(MK012093, MN267831, MK012099, MK012098, MK012095, MK012094, MK012091, MK012092, MK704514, AF179871, MK704513, KX270388, KX270365, KX270387, KX270378, KX270367, KY514162, KY514164, MN275919, MN275910, EU348881, MW883447, MW883448, MN275988, MN275908, MN275907, MH744807, KU748884, KU748883, KU748882, KT266792, KT266791, KU672635) in the GenBank database was supported in the species determination by utilizing the BLAST Algorithm (https://blast.ncbi.nlm.nih.gov/). Alignments of the sequences is performed by using Clustal W (version 5.6.1, 2009, CLC bio, Aarhus, Denmark) and the identity matrix options of Bioedit (Hall, 1999, Hall et al., 2011), respectively. The neighbouring MEGA Version 7 program was used to build a phylogenetic tree (Figure 4.3). The result agreed with (Arefkhah et al., 2020b), which revealed that the phylogenetic analysis by the *B1 gene* was categorized taxonomically as a single clade. Various geographic conditions, the level of parasite genomic diversity for a given region, the sensitivities and polymorphism of the used genetic markers, and the number of polymorphism loci may all have an impact on the variances in genotype in different parts of the world (Rouatbi et al., 2019, Liang et al., 2022).

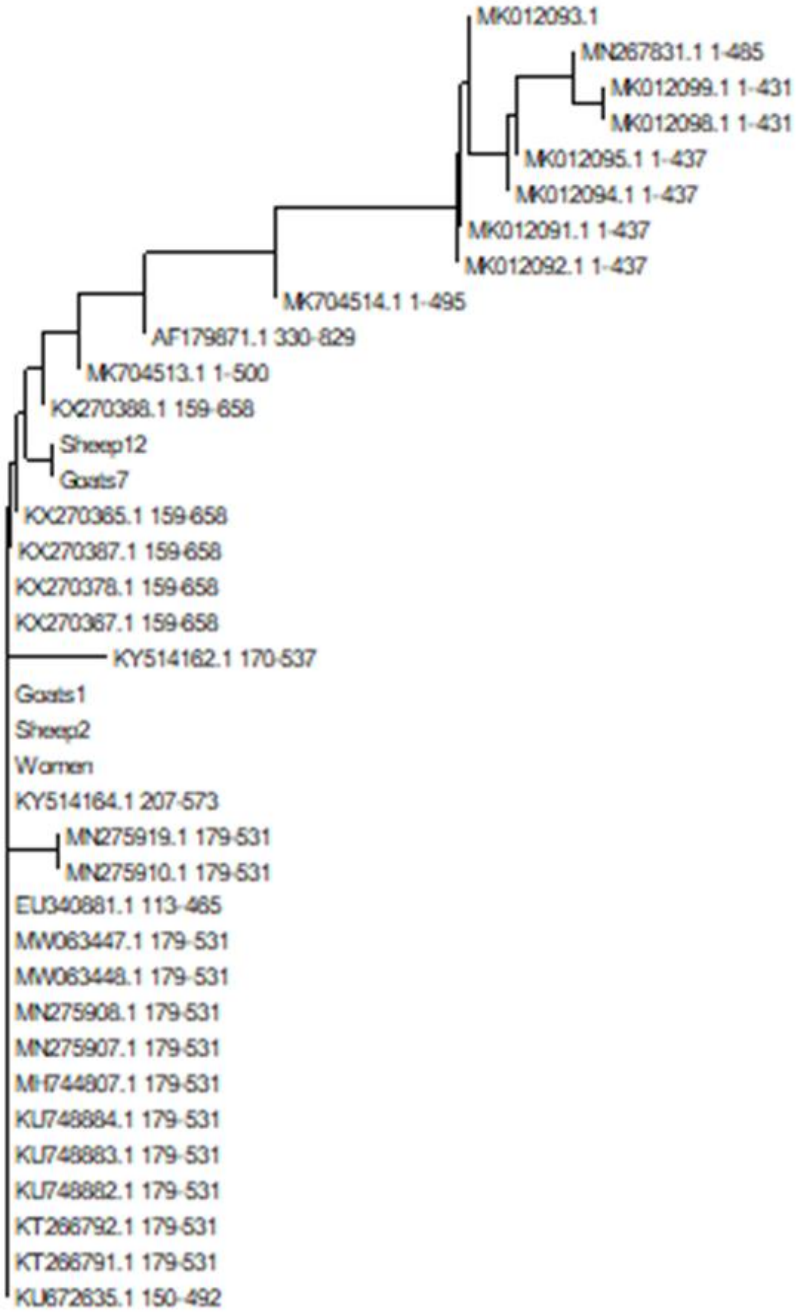

Figure (4.3): Phylogenetic tree based on the *B1 gene* of *T. gondii* isolates from naturally infected women, sheep and goats with accession numbers which previously deposited in the GenBank database.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In the light of the current study. It has been concluded:

1. A higher ratio of toxoplasmosis can be detected by serologic test (cobas e 411) than by polymerase chain reaction (PCR).
2. The seropositivity of anti *T. gondii* IgG antibodies higher than anti *T. gondii* IgM antibodies in women, ewes and does.
3. Seropositivity of *T. gondii* in women who live in rural areas was extremely higher than those who live in urban areas.
4. Higher seropositivity of anti *T. gondii* antibodies was recorded in the illiterate group as compared to the literacy group.
5. The seroprevalence was observed higher in the age range between 20 to 29 years than other age groups.
6. The first trimester recorded higher rate of anti-toxoplasma antibodies IgG and IgM in comparison to second trimester and the lowest rate was found in the third trimester.
7. Women who had contact with cats had a higher seropositivity of anti *T. gondii* antibodies as compared to those who had no contact with cats.

5.2 Recommendation

1. Pregnant women should be screened for *Toxoplasmosis*.
2. Molecular detection of virulence genes of *T. gondii* could be crucial.
3. Further studies to investigate the seroprevalence of anti-toxoplasma antibodies in cats to indicate their role in disease transmission.
4. Furthermore, conduction of seroprevalence of anti-toxoplasma antibodies in other species of domestic livestock.
5. Isolation of *T. gondii* from aborted women is very necessary exploit in laboratory animals (Mice) as one of the confirmatory diagnosis.

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APPENDIX

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By

Hamad Mustafa Saleh[1] and Zuber Ismael Hassan[2]

[1,2] Department of Medical Laboratory Technology, Erbil Health and Medical Technical College, Erbil Polytechnic University, Kurdistan Region/Iraq.

hamad.mstafa@epu.edu.iq

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پوختە

لەم تـوێژینەوەیە هەسـتاین بە لیکـۆلینەوە لە مشـەخۆری دەردە پشـیلە *Toxoplasma gondii* کە دەبیتە هۆی لەباچوون لە ئافرەتـان و مەر و بـزن لە شـاری هەولێـر، هەرێمـی کوردسـتانی عیـراق لـة رێگـای پشـکنینی خـوێن و گەردیـلەی ئەم نەخۆشـیە لە زۆربەی شـوێنەکان بەدی دەکریـت و لە رێگـای ئاژەلەکـان دەگوازرێتەوە. راپرسیەکە کرا بە بەشداربوونی ٨٠ ئافرەتی لەبـارچوو لە هەردوو نەخۆشخانەکانی هەولێـر و سۆران کە پشکنینی خـوێن و گەردیلەیـان بـۆکرا لەمـاوەی تشـرینی دووەمـی ٢٠٢١ تـا مـانگی نیسـانی ٢٠٢٢، لەلایەکیـدییەوە ٥٥ مەر و ٣٠ بزنـی لەبـارچوو بشـدار بـوون لە تـوێژینەوەکە لە هەمـان شـویندا کە نـاویـان هـاتوە و پشکنینی خوێن و گەردیلەییان بۆ کرا. رێژەی بلاوبوونەوەی مشەخۆرەکە لەنـاو ئافرەتـانـدا لەبـارچوو بەم شێوەیە بـوو ٨٠/١٨ (٢٢.٥) بـۆ دژە تەنـی IgG پۆزەتیـف بـوون ئەوانیتـر نەگەتیـف بـوون لەلایەکیـدی ٨٠/٤ (٥٪) بـۆ دژەتەنـی IgM پۆزەتیـف بـووا ئەوانیتـر نەگەتیـف بـوون. جگە لەوەش رێـژەی بلاوبـوونەوەی مشەخۆرەکە لەنـاو مەرەکـانـدا بەم شیوەیە بوو ٥٥/١٣ (٢٣.٦٣٪) بـۆ دژەتەنـی IgG پۆزەتیـف بـوون ئەوانیتـر نەگەتیف بوون جگەلەوە بە رێـژەی ٥٥/٢ (٣.٦٣٪) بـۆدژە تەنـی IgM پۆزەتیـف بـوون ئەوانیتـر نەگەتیـف بوون. سەرەرای ئەوەش رێژەی بلاوبـوونەوەی مشـەخۆرەکە لەنـاو بزنەکـانـدا بەم شێوەیە بـوو ٣٠/٨ (٢٦.٦٦٪) بـۆ دژەتەنـی IgG پۆزەتیـف بـوون ئەوانیتـر نەگەتیـف بـوون جـگەلەوەش بە رێـژەی ٣٠/١ (٣.٣٣٪) بـۆ دژەتەنـی IgM پۆزەتیـف بـوون ئەوانیتـر نەگەتیـف بـوون.لیکـۆلینەوە گەردیلەیەکـان بـاش گەورەکردنیـان دەریـان خست کە نموونەکان ١٠٠٪ مشـەخۆری *Toxoplasma gondii* بـوون. ئەنجامەکـانی تـوێژینەوەکە دەریخسـت کە هەر یەک لە نموونەکـانی(ئـافرەتی ژمـارە ١، مەری ژمـارە ٢، وە بزنـی ژمـارە ١) ١٠٠٪ مشەخۆرەکە بوون وە مەری ژمارە ١٢ و بزنی ژمـارە ٧ بەرێـژەی ٩٩.٩ ٪ هاوشێوەی *T.gondii* بـوون لە ژیـر ژمـارەی پەیوەستبوون (KX270387 و MK704513)بەهـۆی جێگـرەوەی تـرشـە ئەمینـی (A→G)لە شوێنی ٢٠٧ دا.

سیرۆبریۆالینس (ڕێژەی بڵاوبوونەوەی) و دیاریکردنی گەردیلەیی بۆ مشەخۆری *Toxoplasma gondii* لە ئافرەتی لەبارچوو، مەر و بزن لە پارێزگای هەولێر

نامەیەکە

پێشکەش بە ئەنجومەنی کۆلێژی تەکنیکی تەندروستی هەولێر کراوە لە زانکۆی پۆلیتەکنیکی – هەولێر وەک بەشێک لە پێداویستیەکانی بەدەستهێنانی پلەی ماستەر لە بەشی شیکاری نەخۆشیەکان

لە لایەن

حمد مصطفی صالح

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پرۆفیسۆر یاریدەدەری دکتۆر زوبیر اسماعیل حسن

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