Republic of Iraq
Ministry of Higher Education
And Scientific Research
University of Misan
College of Science

Molecular Detection and Immunohistological Changes of Placenta Caused by *Toxoplasma gondii* in Aborted Women in Maysan Province, Iraq.

A Thesis
Submitted to the College of Science, University of Misan in
Partial Fulfillment of the Requirements for
M.Sc Degree *in Biology*

BY

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B.Sc. in Biology / College of Science / University of Misan (2019)

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2023 A.D **1444 A.H**

بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

هُوَ الَّذِي يُصَوِّرُكُمْ فِي الْأَرْحَامِ كَيْفَ يَشَاءُ ۚ لَا إِلَٰهَ إِلَّا هُوَ الْعَزِيزُ الْحَكِيمُ

صدق الله العظيم

سورة آل عمران اية 6

Supervisor Certification

We certify that this Thesis, **(Molecular Detection and Immunohistological Changes of Placenta Caused by *Toxoplasma gondii* in Aborted Women in Maysan Province, Iraq.)** Submitted by (Safaa Salman Dawood) was prepared under our supervision at the Department of Biology / College of Science / University of Misan, as a partial fulfilment of the requirements for the degree of Master of Science *in Biology*.

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In view of the available recommendations, I forward this thesis for debate by the examining committee.

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4 / 5 / 2023

Dedication:

To my father and my mother who encouraged and motivated me to complete my study, and without their love and support, finishing this work would not have been possible.

Safaa

Acknowledgments:

Firstly, I thank **God** who enabled me to complete my thesis, surrounded me with his mercy and care, and inspired me with patience throughout my academic journey .

My sincere thanks to Prof. Dr. **Hussain Ali Mhouse AL-Saady** who supervised me and gave me much useful advice, and for suggesting the title of the thesis and allowing me to benefit from his experiences.

I would like to thank the **Dean** of the College of Science, University of Misan, and my thanks extended to the head of the Department of Biology **Dr. Maytham Abdul Kadhim Dragh** , and all the **teaching staff** for their cooperation and encouragement.

I especially thank the medical **staff at Al-Sadr Teaching Hospital** for their cooperation and assistance me in collecting samples for this study. I want to express my gratitude and thanks to all **the researchers** over the world whom I mentioned in my thesis.

All my gratitude to **Dr. Maan Mohsen Neamah, Dr. Hudhayfa Abdul Hussain Ali, and Dr. Muhammad Talib Muhammad** in U-gene laboratory in Al-Najaf province for helping me in the practical part and giving me their notes .

Finally, I express my deep gratitude to my dear parents, **Salman** and **Abtsam**, who set a wonderful example of support, and to **all my friends** who did not hesitate to ask about me and give me positive energy in the hardest times.

Safaa

Summary:

This study was conducted to investigate the relationship between toxoplasmosis and the abortion in women who were admitted to Al-Sadr Teaching Hospital, in Maysan province, Iraq, from November 2021 to May 2022.

This study has been conducted using five different methods to check their ability including serological lateral flow immunoassay (LFIA), immunohistochemistry (IHC) histopathology (HP), Real-Time quantitative Polymerases Chain Reactions (RT-qPCR), and Restriction Fragment Length Polymorphism (RFLP) technique to identify the genotypes of *Toxoplasma gondii* parasite.

It was found that these methods are vary in their ability to detect the parasite, LFIA 55%, IHC 80%, HP 28% and RT-qPCR 10% for placenta tissues and 0% for the blood samples, and found that genotype "I" is the most frequent than genotypes II and III (50%, 33.33%, and 16.67%) respectively. The present study showed that IHC was the most sensitive and reliable test.

The sectioned tissuse that have been examined by IHC and HP were revealed that the infection with *T. gondii* is has caused severe damage including necrosis, destruction in infected tissues and blood vessels, hemorrhage, granuloma, and fibrosis in the decidua, stroma, trophoblast, and villi of the placenta, with a high density of *T. gondii* stages in the site of infections.

The study concludes that there is a significant relationship between infection with *T. gondii* parasite and abortion in women.

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List of abbreviations

Abbreviation	Meaning
AIDS	Acquired Immunodeficiency Syndrome
Ca^{+2}	Calcium ion
$CD4^{+}$	Cluster of Differentiation 4
CT	Congenital Toxoplasmosis
DAB+	3,3'-Diaminobenzidine
DDW	Double Distilled Water
DH	Definitive Host
DNA	Deoxyribonucleic Acid
dNK	decidual Natural Killer
EDTA tube	Ethylene Diamine Tetra Acetic Acid Tube
ELISA	Enzyme-Linked Immunosorbent Assay
EVT	Extravillous Trophoblasts
GRA6	dense granule protein 6
HCG	Human Chorionic Gonadotrophin
HP	Histopathology
HPL	Human Placental Lactogen
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IH	Intermediated Host
IHC	Immunohistochemistry
IL-12/IL-10	Interleukin-12/ Interleukin-10
LAT	Late Agglutination Test
LEAs	Late Embryogenesis Abundant proteins
LFIA	Lateral Flow Immunoassay
M1	Classically-activated macrophages

M2	Alternatively-activated macrophages
MFI	Maternal-Fetal Interface
min	Minute
MJ	Moving Junction
OT	Ocular Toxoplasmosis
PCR	Polymerase Chain Reaction
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuole Membrane
PYR	Pyrimethamine
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
SDZ	Sulfadiazine
sec	Second
SFDT	Sabin Feldman Dye Test
spp.	Species
SPSS	Statistical Package for the Social Sciences
SYN	Syncytiotrophoblasts
T. gondii	*Toxoplasma gondii*
TBS	Tris Buffered Saline
TNF-α	Tumor Necrosis Factor alpha

CHAPTER ONE

Introduction

1.Introduction:

The parasites are widely spread all around the world and have a significant role in human disease (Cox, 2004). Protozoan are causing important human and animal diseases which include the phylum: Apicomlexa, which contains many intracellular parasites such as *Toxoplasma gondii, Plasmodium spp., Eimeria spp.,* and others (Corvi *et al.,* 2012). *T. gondii* is deemed one of the most prosperous parasites in the world because it is a polyxenous protozoan (Tenter *et al.,* 2000). It infects the nucleated cells of about 350 species of endotherm animals as an intermediate host (IH), while domestic and wild feline are the definitive host (DH) (Hatam-Nahavandi *et al*., 2021).

Toxoplasmosis infected about a third of the human population (about 2.5 billion) in all regions of the world and the majority of immunocompetent had asymptomatic diseases (Schultz and Carruthers, 2018). *T. gondii* causes serious diseases in immunocompromised individuals and it may lead to blindness and mental retardation in fetuses and newborns (Hill and Dubey, 2002).

Herbivores acquire infection by ingesting weeds or water contaminated with oocysts that excreted with cat feces to an external environment, while carnivores get infection mostly by feeding on prey meat (IH) containing tissue cysts (bradyzoite), human-acquired infection by both routes (Hill and Dubey, 2018; Almeria and Dubey, 2021). Humans can get the infection through taking undercooked meat, unpasteurized milk, blood transfusion, and organ transplant (Pinto-Ferreira *et al*., 2019; Mijbel and Alsaady, 2022). The parasite invades the host cells and establishes a shelter inside them and

continues to divide until the cell explodes the parasite attacks the rest neighboring cells, forming a lytic cycle, where *T. gondii* remodels the host cell leading to an increase in the pathogenesis, which represented by increasing the migratory activity of the infected cell to facilitate its spreading (Blader *et al*., 2015; August *et al.,* 2020).

In a pregnant woman, when it infected the *T. gondii* orally for the first time (primary infection), the parasite transforms from the pathogenic stage to the quickly multiplying stage (tachyzoite), this stage spreads to the blood and then to all parts of the body tremendously and it can be passed from the mother to her fetus through the placenta tissue and caused congenital toxoplasmosis (Barragan and Sibley, 2003; McAuley, 2014). When a pregnant woman is infected with *T. gondii* in the first trimester, the rate of birth of congenitally deformed children and stillborn is high, and this parasite is classified as one of the most dangerous parasites in the pregnancy period (Al-Hindi, 2009). Through the acute phase of infection, which does not exceed ten days, the parasite faces a strong immune response for the spreading of the parasite throughout the body is reduced, but *T. gondii* adapts to this immune response by turning into the resistant phase (bradyzoite) that cysts in the muscles and the brain (Filisetti and Candolfi, 2004).

It is necessary to raise awareness and early diagnosis of toxoplasmosis with highly sensitive tests, like Polymerase chain reaction (PCR) or serological tests (Al-Safar *et al*., 2019). The Immunohistochemistry method was used to detect the anti-*Toxoplasma gondii*, specifically in the villous trophoblast, which is found in the human placenta, because it is characterized by high sensitivity and specificity (Huppertz and

Gauster, 2011). The serological tests are quick and widely available which contributes greatly to detection the infection earlier (Liu *et al.*, 2015).

The early treatment of toxoplasmosis can reduce depression in pregnant women, abortion cases, and the economic impact of the parasite, such as the treatment cost of infected infants who suffers from blind and mentally retarded (Paquet *et al.*, 2013; Nourollahpour Shiadeh *et al.*, 2016).

1:1 The aim of the study:

The study aimed to investigate the role of the women placenta in the transmission of *T. gondii* from the mother to her fetus, and influence of toxoplasmosis in abortion fetus by some objectives :

1. The use of immunological methods to detect *T. gondii* parasite in the blood of women participating in the study.
2. The use of RT-qPCR to detect the *T. gondii* in blood and placenta tissue.
3. The use of IHC technique to detect the *T. gondii* in the placenta tissues.
4. The use of histopathological technique to detect the damage that may cause by *T. gondii* in the placenta of aborted women compare with non-infected and non-aborted women.
5. Using restriction Fragment Length Polymorphism (RFLP) PCR to identify the genotype of *T. gondii* in Maysan province.

CHAPTER TWO

Literatures Review

2.Literatures Review

2:1 The history of *Toxoplasma gondii,* the discovery and clinical observations:

Toxoplasma gondii was discovered in 1908 by Nicolle and Manceaux by chance in a desert rodent, *Ctenodactylus gundi*, at the Pasteur Institute in Tunisia, they gave the species name of the parasite from the species name of that rodent, and then the genus was named *Toxoplasma* wherein Greek world mean (toxon = arc, plasma =form), this is derived from trophozoite shape (tachyzoite) which likes a crescent shape (Frenkel, 1970; Roberts and Schimidt, 2000; Hill *et al.,* 2005). At the same time, *T. gondii* was shown in the tissues of a rabbit in Brazil by Splendore (Weiss and Dubey, 2009).

The parasite pathogenicity was confided in 1939 by Wolf, Cowen, and Paige who detected the parasite in the tissues of a child with congenital infection, and many reporters mentioned to clinical symptoms of toxoplasmosis, but without recognizing the main cause so considered it as symptoms of unknown cause (Dubey, 2008). *T. gondii* has been a mystery to researchers for over half a century since 1970 when were discovered that felines as the final host (Dubey, 2009). The most famous incident in history was in 1966 when 99 students were infected with *Toxoplasma* at the University of São Paulo College of Medicine in Brazil after three months when they ate an undercooked meat hamburger (Magaldi *et al.,* 1969). The first record of toxoplasmosis in Iraq was in 1938 by Machattie, who showed it in the tissue of two dogs in Baghdad (Machattie, 1938).

The Figure 2-1 summarized the most prominent historical events related to the parasite:

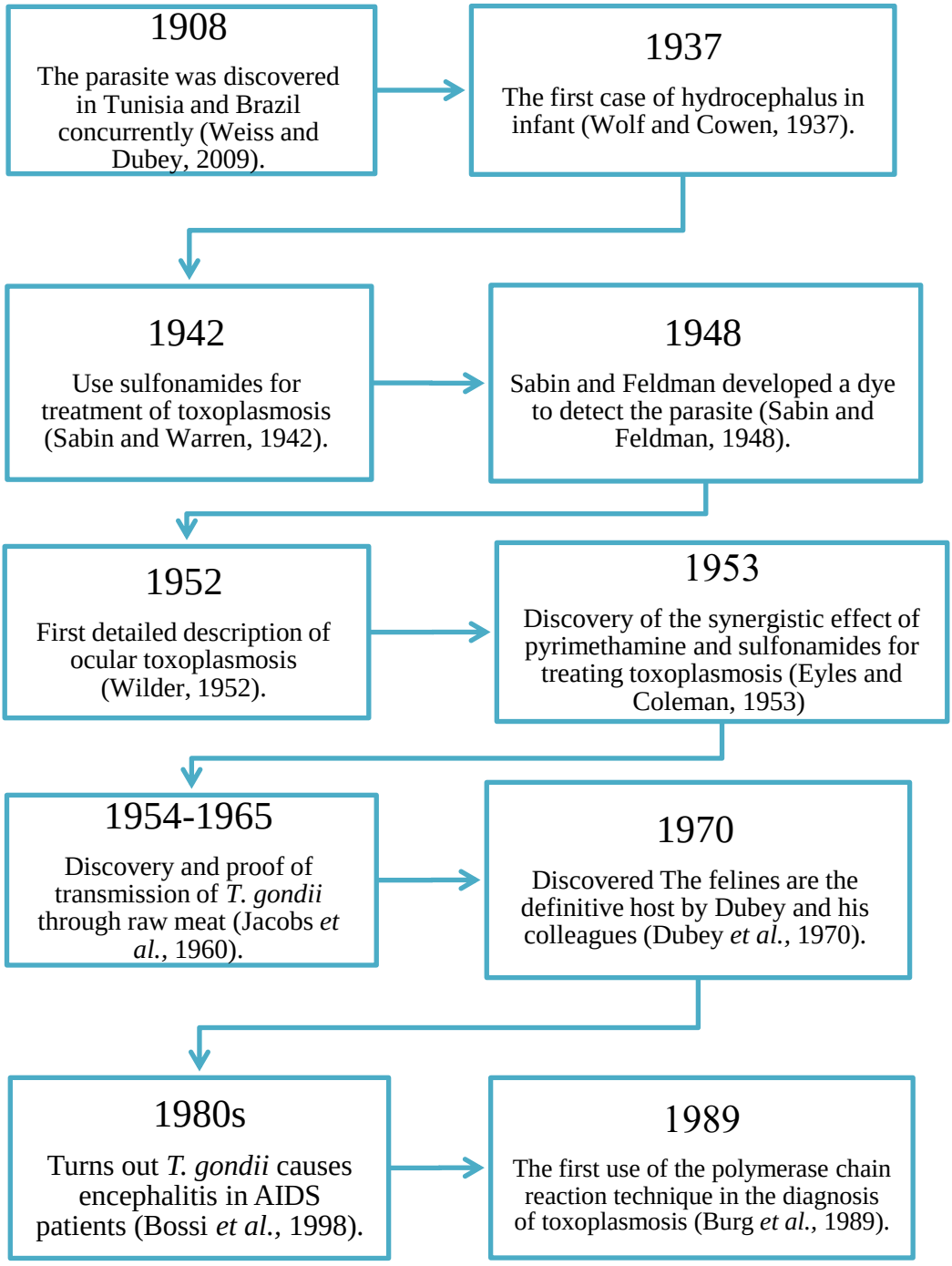

Figure 2-1: An outline of the most important discoveries in the history of *Toxoplasma gondii*.

2:2 Classification of *T. gondii*:

The parasite was classified according to Hill and Dubey (2014) as follows:

Kingdom : Protista

Phylum : Apicomplexa

Class: Sporozoasida

Sub class : Coccidiasina

Order : Eucoccidiorida

Sub order : Emeriorina

Family: Toxoplasmatidae

Genus: *Toxoplasma*

Species: *Toxoplasma gondii*

By (Nicolle and Manceaux, 1909)

2:3 Morphology of *T. gondii*:

2:3:1 life cycle stages:

2:3:1:1 Tachyzoites:

The tachyzoites stage was named by Frenkel based on the rate at the cells divide, from the Greek word "tachos" means speed, and it is also called endozoites or endodyozoites and "zoon" mean animal, the tachyzoite is an alternative term to the trophozoite (Dubey *et al*., 1998; Smith and Evans, 2009). Tachyzoites are ranged between (4-8 x 2-4) μm (long x wide), and their shape varies from oval to a crescent (Fig. 2-2) (Montoya and Liesenfeld, 2004). It is the representative stage of

T. gondii that occurs during acute infection and before the immune response of the intermediate host has developed (Filisetti and Candolfi, 2004; Tu and Weiss, 2018).

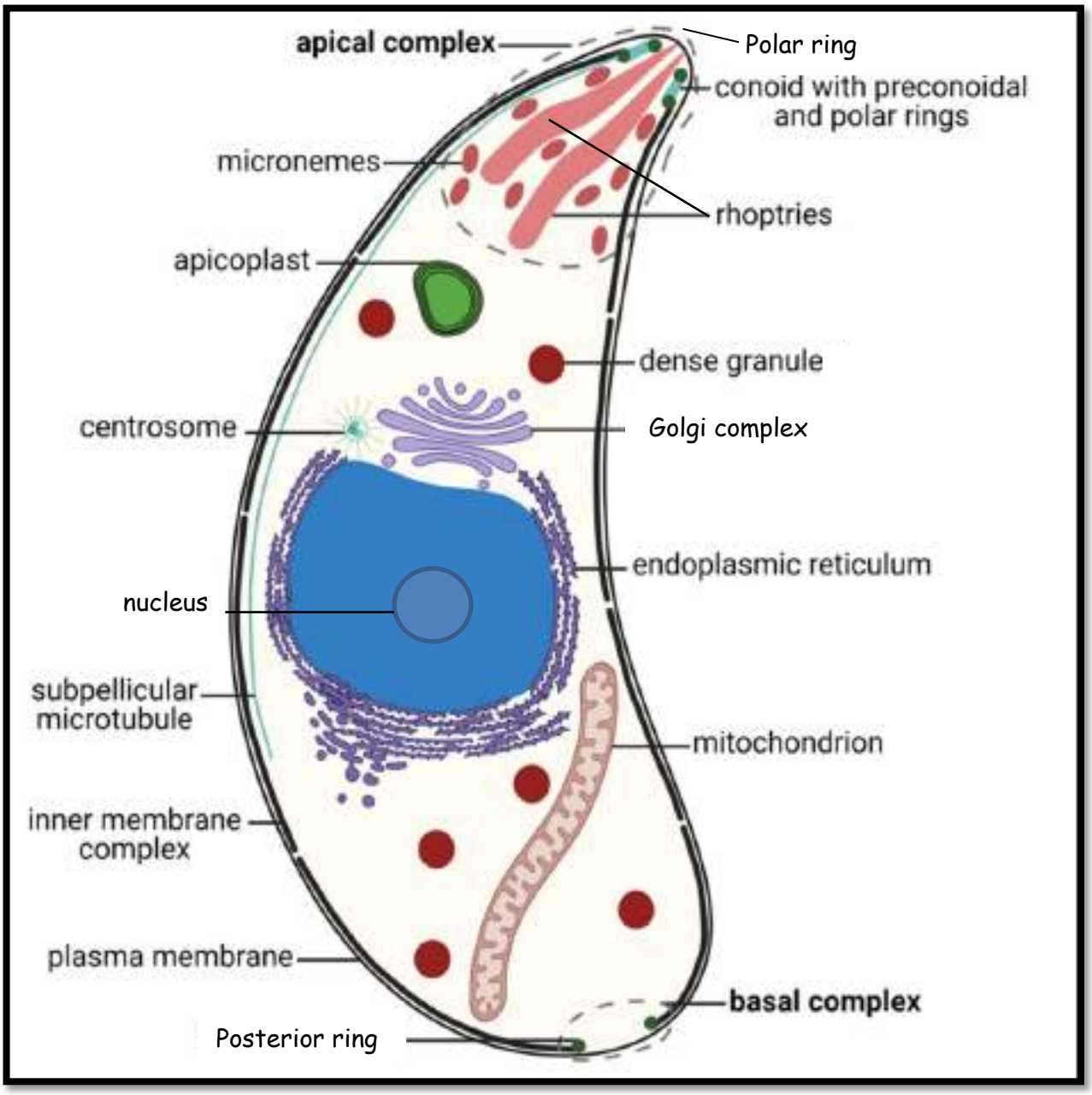

Figure 2-2 : Tachyzoite stage (Delgado *et al.*, 2022).

The *T. gondii* anterior is pointed and its posterior end is around and contains many organelles, the nucleus is central, and it is worth to

mention that the parasite is devoid of pseudopodia, cilia, and flagella, but moves by slipping, undulation, and flexion (Dubey, 2016).

Tachyzoite is relativity incapable of tolerant stomach secretion, therefor, it is less critical for infection than *T. gondii* in other stages (Roberts and Janovy, 2009).

2:3:1:2 Bradyzoite (Pseudocyst):

The bradyzoite stage (Fig.2-3) comes successively after the tachyzoite turns inactive in the tissue as a (tissue cyst) it is considered a smart tactic used by the parasite to move asexually from its different intermediate hosts without passing through a sexual cycle (Sullivan and Jeffers, 2012). Bradyzoites remain inactive and slow to multiply inside the tissue cyst, and usually, the newly formed tissue cysts contain less number of bradyzoites stages compared to the old tissue cyst includes thousands of them (Dubey, 2016).

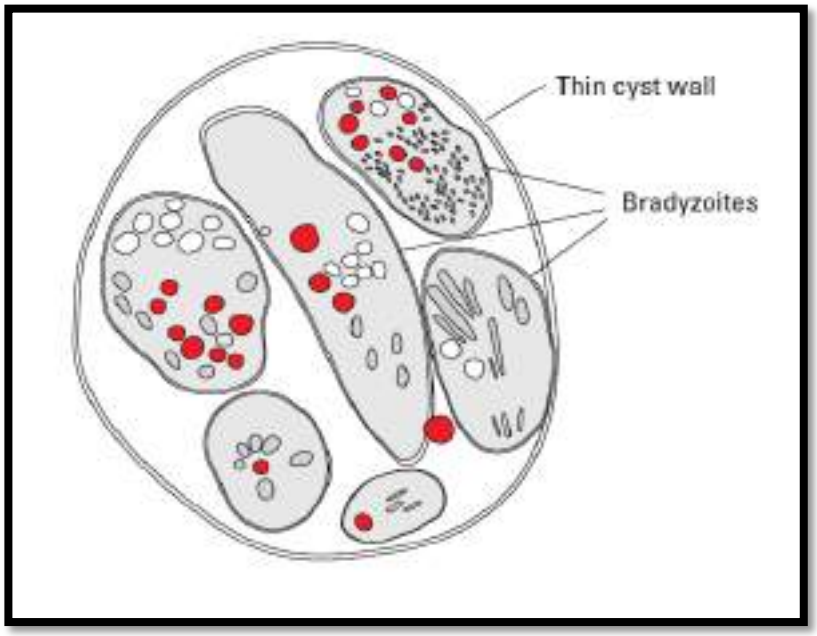

Figure 2-3 : Bradyzoite stage (Hill and Dubey, 2014).

2:3:1:3 Oocyst:

The oocyst stage is produced by the sexual reproductive from the fertilization of macrogametes with microgametes inside the epithelial cells of the intestine of a feline (Tomasina and Francia, 2020). Surrounded by a solid protective wall, it consists of two colorless layers, that cover the oocyst from being directly exposed to extreme environmental conditions (Gagne, 2001; Salman *et al*., 2017). Among a large number of proteins that inter in the structure of the oocyst wall the late embryogenesis domain-containing proteins (LEAs) that keep oocysts survival and resistant to desiccation (Fritz *et al*., 2012).

The shape of young oocysts is spherical with a diameter of 10-12 μm while the mature ones have a semi-spherical shape with a diameter of 11-13 μm, each mature oocyst had two sporocysts, and each sporocyst 6-8 μm in diameter had four sporozoites (Fig. 2-4) (Dubey *et al*., 1998).

The mature oocyst and their sporozoites can be survived infectively at a temperature of 4° C for four and a half years in fresh water, two years in salt water (causing toxoplasmosis in marine animals) and one year in wet soil (Dubey, 1998; Lindsay and Dubey, 2009). Humidity and temperature are determining factors for the survival of oocysts in the soil and their ability to infect, and the areas most contaminated with oocysts are parks and public gardens (Gao *et al*., 2016; Saki *et al.*, 2017).

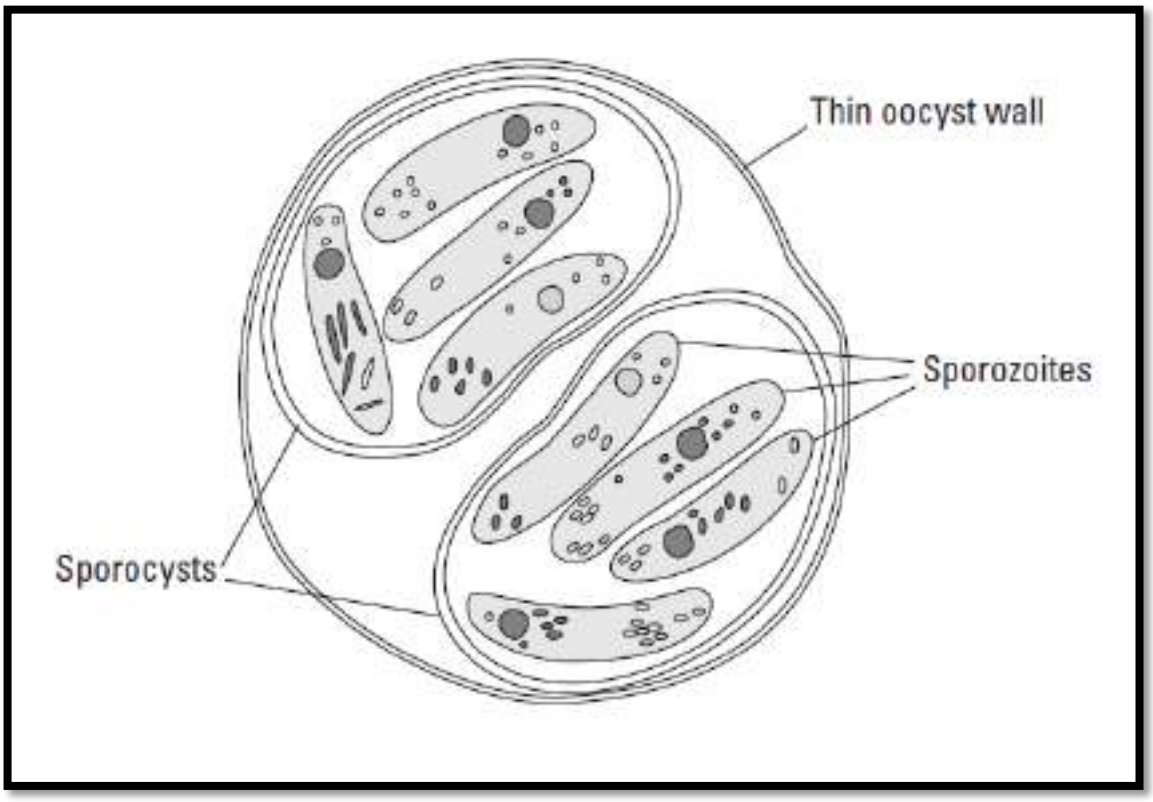

Figure 2-4 : Oocyst stage (Hill and Dubey, 2014).

2:3:2 An important structure in the life cycle of *T. gondii*:

2:3:2:1 The Parasitophorous Vacuole:

Parasitophorouse vacuole (rose-like arrangement) it is a temporary structure of *T. gondii* which take the host cell plasma membrane as physically a separated bubble of cytoplasm (Ferreira-da-Silva *et al*., 2010; Clough and Frickel, 2017).

When the tachyzoite penetrates the host cell, it exploits the cell membrane as a wall around it, forming what is called parasitophorous vacuole membrane (PVM) (Fig. 2-6), but after some time the parasite changes the host's proteins with its proteins so that it becomes a single entity in the cell, the members of apicomplexa use the PVM as a protective barrier within the host cells from attacking of the host

cell lysosomes through different mechanisms and it allows the parasite to survive (Beyer *et al*,. 2002).

The PVM is a unique and dynamic "organelle" that can only be found within the infected cell, see (Fig. 2-5) (Martin *et al*., 2007). It is also responsible for the special functions of obtaining nutrients and maintaining the vacuole structure (Sinai, 2008).

The calcium ionosphere and the extracellular calcium chelate play important role in the inhibition of the host cells invasion and replication of *T. gondii* inside it (Song *et al.*,2004). As well as the cytosolic Ca^{+2} can act as a regulator of the trigger egress of *T. gondii* (Vella *et al.*, 2021).

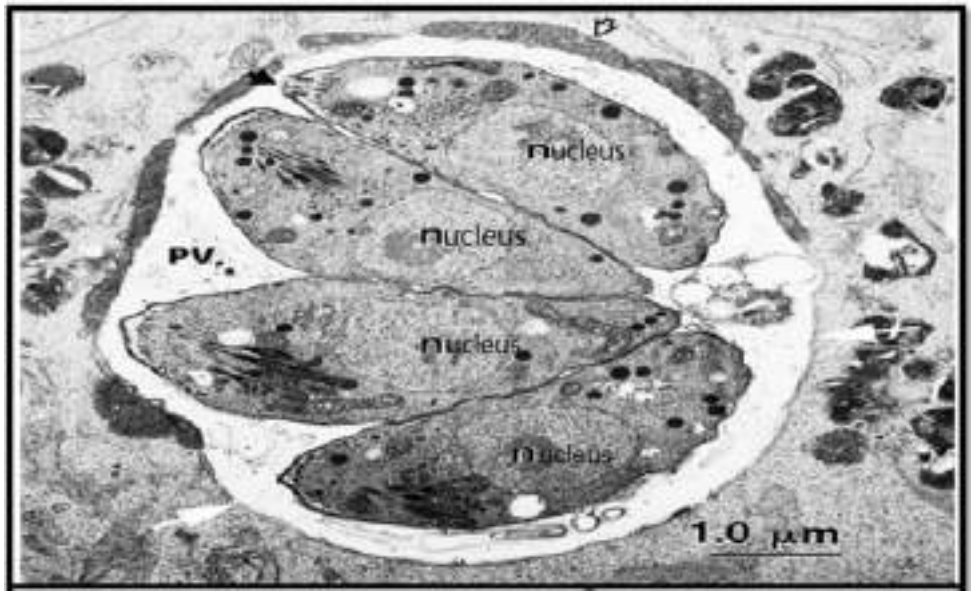

Figure 2-5: The parasitophorous vacuole (PV) within host cells (Peng *et al.*, 2011).

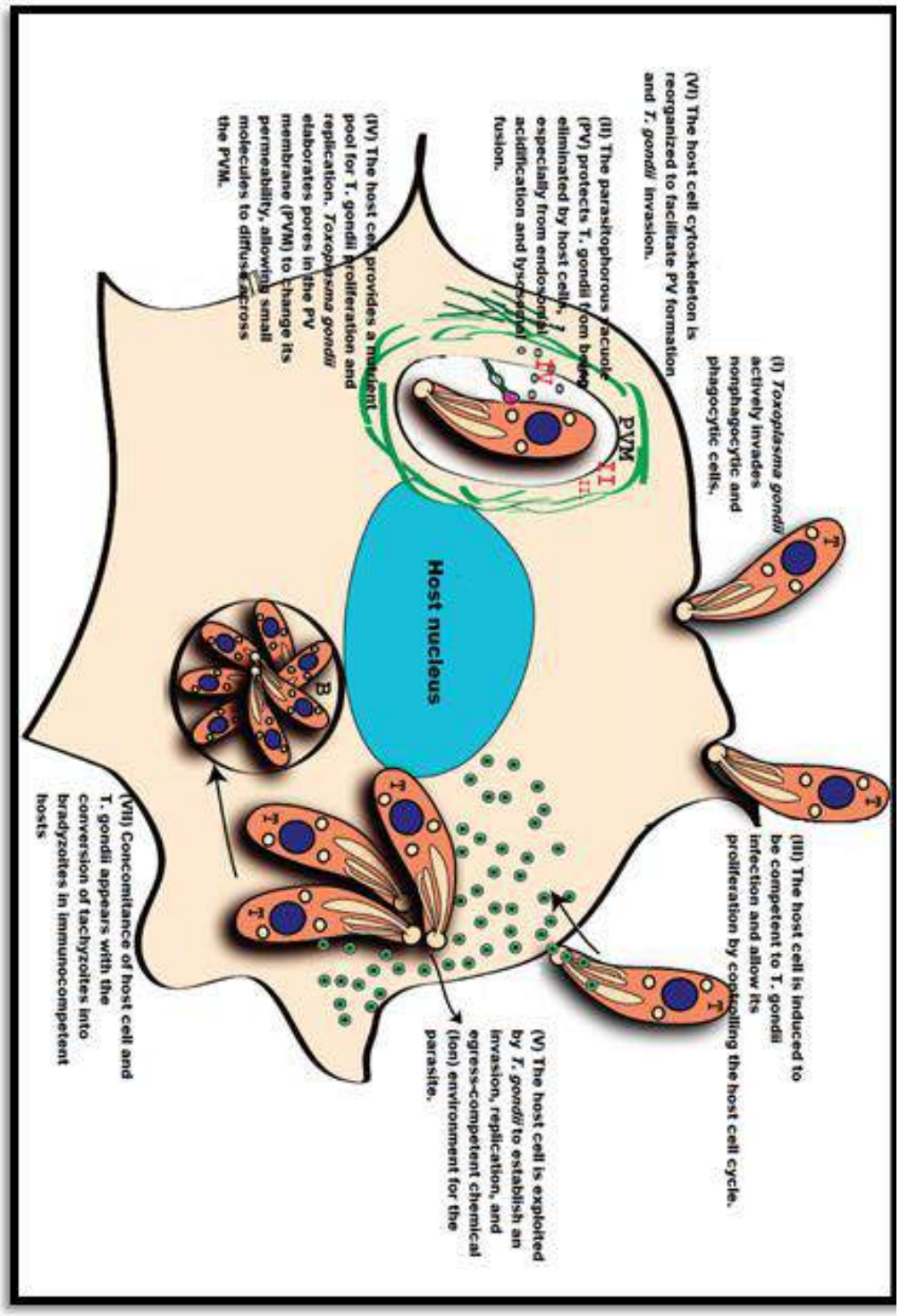

Figure 2-6 : Diagram showing tachyzoites invasion of the host cell and parasitophorous vacuole formation, with modification (Peng *et al.*, 2011).

2:3:2:2 Endodyogeny:

Endodyogeny in greek is defined as endo means inside or internal, dyo means dual and geny means genesis, it is a complex process of asexual reproduction methods of parasites, such as tachyzoites in *T. gondii*, which leads to forming two new cells in the mother cell (van der Zypen and Piekarski, 1967; Smith , 2010; Gubbels *et al*., 2020).

The two cells develop to take a horseshoe shape, and the nucleus and cytoplasm of the mother cell are divided between the new two daughter cells, then the mother cell disappears to replace by budding bodies of the two new cells (Fig. 2-7) (Sheffield and Melton, 1968).

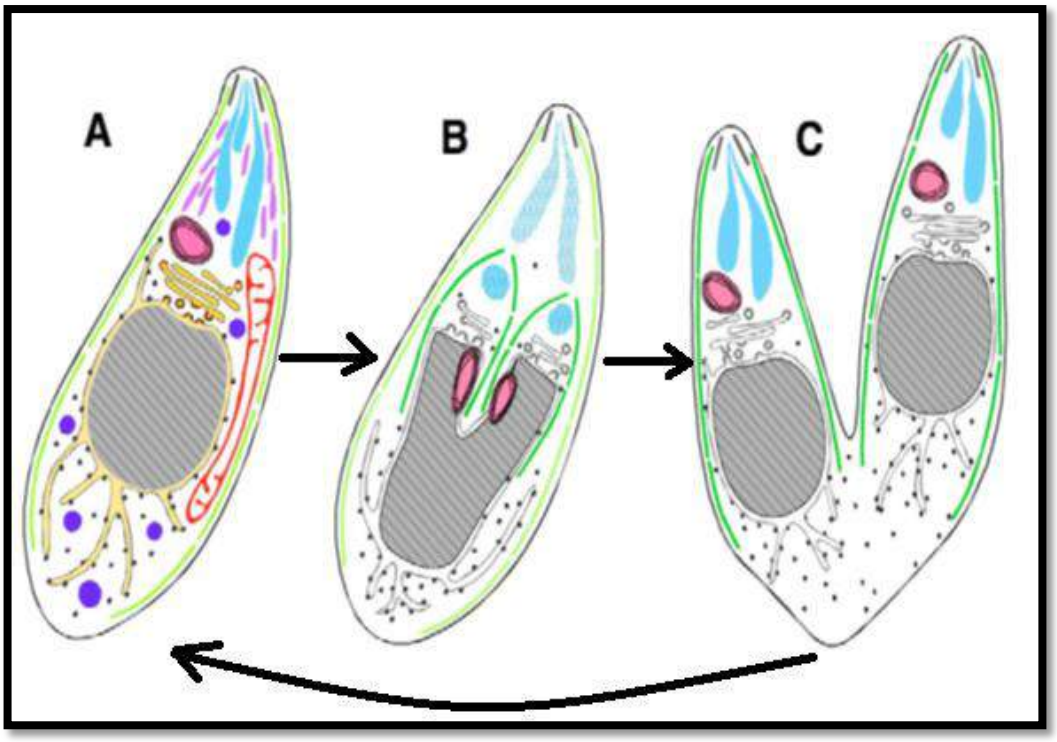

Figure 2-7: Endodyogeny in *Toxoplasma gondii* (Nishi *et al*., 2008).

2:3:3 The life cycle of *T. gondii*:

The complete life cycle of *T. gondii* had remained unknown for more than six decades until discovery it in the cat are the defined hosts (Ferguson, 2022). The life cycle of *T. gondii* includes two cycles, the entero-epithelial cycle and the extra-intestinal cycle (Fig. 2-9).

2:3:3:1 Entero-epithelial cycle (sexual):

The Entero-epithelial cycle begins when one of the feline species feeds on a toxoplasmosis-infected animal such as infected meat, mouse, bird, or any infected endotherms-animal (Dubey and Lindsay, 2004). When the DH ingested the *T. gondii* tissue cyst, the bradyzoites liberate as a result of the breakdown of the tissue cyst wall in the stomach and small intestine by the action of digestive enzymes (Dubey,1996).

The bradyzoites penetrate the cells of the enteroepithelial tissue and then develop and replicate into merozoites stages, and undergo to series of asexual reproduction by merogony, then some merozoites developed into sexual gametes to produce microgamete (male gametes) which are small and each has two flagella, and macrogametes (female gametes) which are large in size and have oval shape (Fig. 2-8) (Garcia, 2006; Ferguson and Dubremetz, 2014).

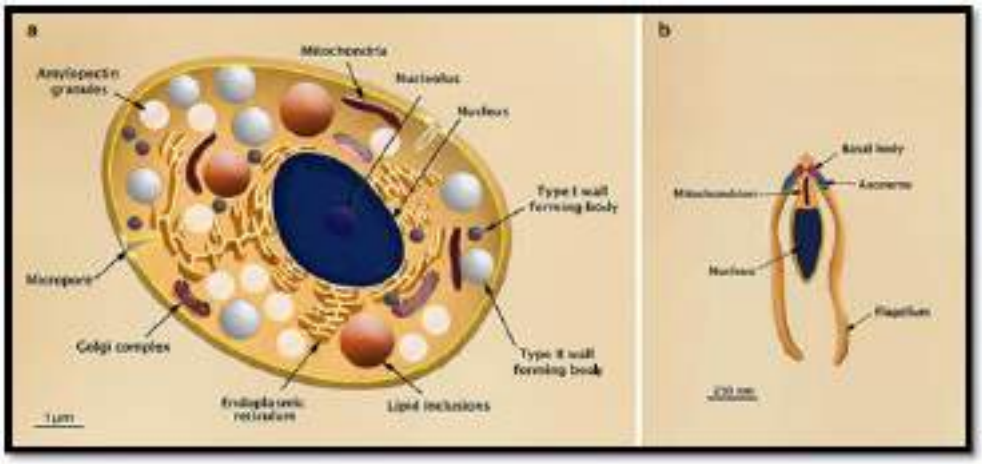

Figure 2-8: *Toxoplasma gondii* gametes (a) the macrogamete and (b) microgamete (Attias *et al.*, 2020).

The parasite is sensitive to linoleic acid levels in the gut, particularly in the ileum, in high levels, the parasite is indicating that the target host is a feline and the sexual cycle begins, since it lacks the enzyme delta-6-desaturase, which converts linoleic acid to arachidonic acid, as act in other animals, therefore it is a remarkable characteristic for the parasite to recognize its host without seeing it (Hofer, 2019).

Then, the fertilization process was done by the egress of the microgamete from the epithelial cell and entering the cell containing the macrogamete to fertilize it, then the macrogamete developed into a zygote and then oogonia and oocyst which surrounded itself by a thick membrane, in each cycle, millions of oocyst are produced which takes about 3-5 days, the infected cat releases the oocyst in its feces for up to a week (Dubey, 1991; Davis and Dubey, 1995).

The excreted oocyst to the external environment with fresh cat feces is unsporulated, it contains a single cell called sporont, this stage is not capable of infecting unless it matured and becames sporulated by the process of sporogony, in which the sporont is divided into two

sporocysts, and each of them divided into four sporozoites (Lappin, 1999). It was found that domestic cats are responsible for the environment contamination with *T. gondii* up to three times more than feral cats (Dabritz *et al*., 2006). Notably, the infected cats do not get infected again with toxoplasmosis, they acquire immunity against the parasite (Gagne, 2001).

2:3:3:2 Extra-intestinal cycle (Asexual):

T. gondii extra-intestinal cycle occurs in the tissue of intermediate hosts as well as felines, where a large number of bradyzoites developed into tachyzoites in the small intestine to begin the sexual cycle whereas other pass to mucosa to complete the asexual cycle in lamina propria, leukocytes in lymph node, other organ such as brain, muscles, heart, liver, lung, eyes and others (Dubey and Frenkel, 1972; Roberts and Janovy, 2009; Smith, 2009).

When the intermediate host feeds on *T. gondii* tissue cyst-contaminated meat, the bradyzoites during digestion released in the intestine lumen and turns into tachyzoites and attacked the entero-epithelial cells to pass through the lamina propria of IH, then started a series of asexual reproduction through endodyogeny to form merozoites by merogony (Tenter *et al*, 2000; Harker *et al* , 2015).

This stage corresponded with the start of the acute phase of infection, it is presented in the blood for only the first 14 days before it turns into bradyzoite (Lyons *et al*., 2001; Gissot, 2022).

T. gondii infects all nucleated cells of endotherms animals but prefers the monocyte set, especially phagocytes, tachyzoite in infected cells

transforms into a round/oval shape which avoids the phagocytosis by creating PV and multiplying inside it and exploits it as Trojan horse to transition to other tissues (Murray, 2011; Hill and Dubey, 2014).

Most tachyzoites are killed by the immune system, but the rest of them evade the body's defence by escaping into the brain and muscle cells and turning into bradyzoites in tissue cysts (Skariah and Mordue, 2010).

The cycle is completed when the felines feed on infected animals, declaring an endless cycle of repetitions (Lainson, 1957). Bradyzoite is a dangerous measure of toxoplasmosis, as it turns into the rapid stage (tachyzoite) again when the human body is exposed to weak immunity for any reason due to advanced age, exposure to disease that suppress the immune system such as Acquired Immunodeficiency Syndrome (AIDS) or by taking immunosuppressive drugs during organ transplantation (Saadatnia and Golkar, 2012; Knoll *et al*, 2014).

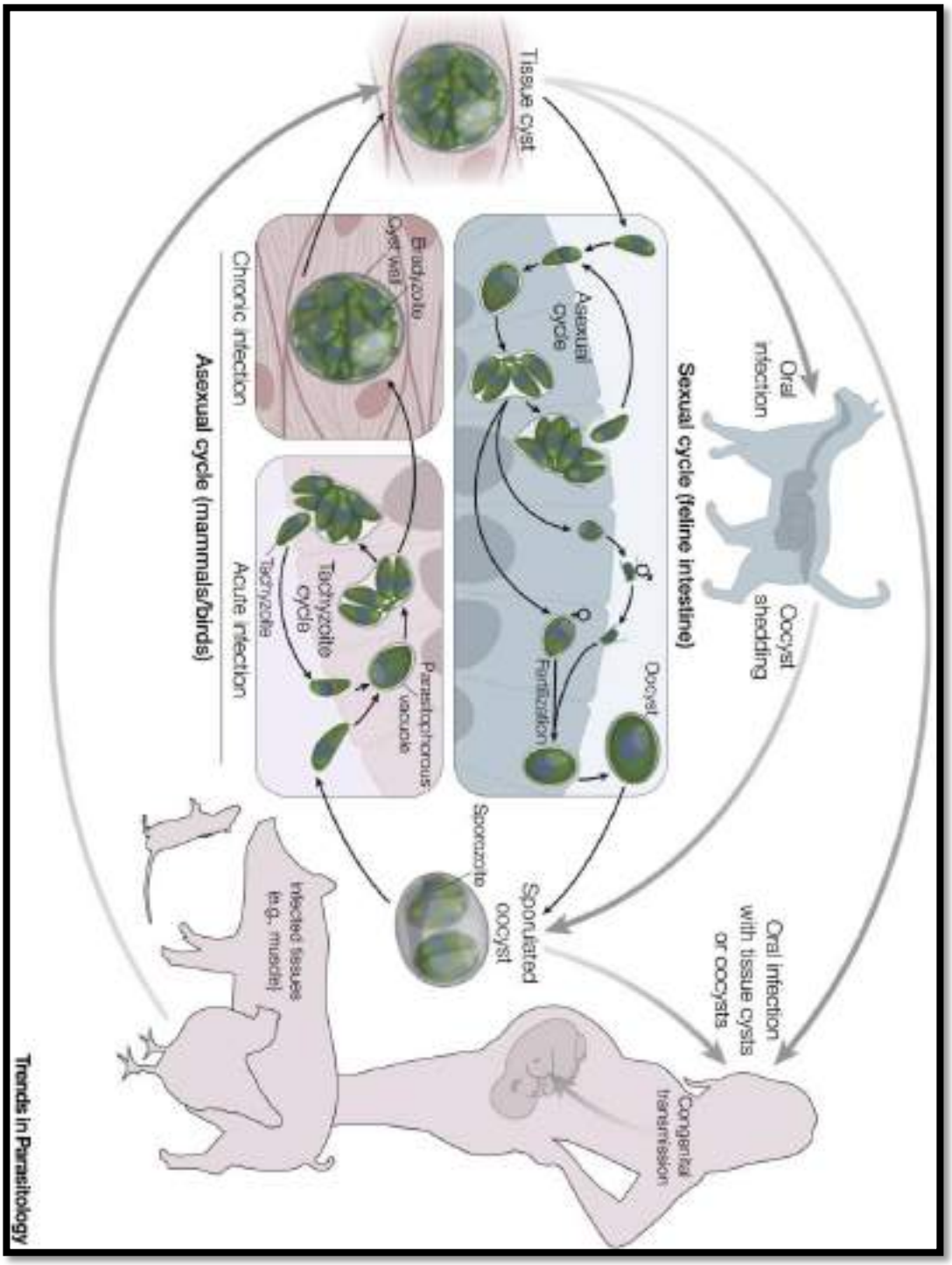

Figure 2-9: Life cycle (sexual and asexual) of *Toxoplasma gondi* (Lourido, 2019).

2:4 The entry and immune evasion:

The traditional way of entering cells is phagocytosis but toxoplasma gondii uses a different way called active penetration by forming a tight-junction contact point between the host and parasite membranes this is known as a Moving Junction (MJ) (Beck *et al*., 2014).

For this tachyzoite secretes a protein complex originating from three secretory organelles collaborate, the first two micronemes and rhoptries, which act at the time of the tachyzoite penetration to the host cell, and the last one is slow to secrete called dense granules (Carruthers and Sibley, 1997).

Rhoptry proteins aid to construct the PV of tachyzoite and this act as an adorable advantage to the parasite for living inside host cells to avoid the immune response and the cell's defences (Carruthers and Boothroyd, 2007; Dubremetz, 2007).

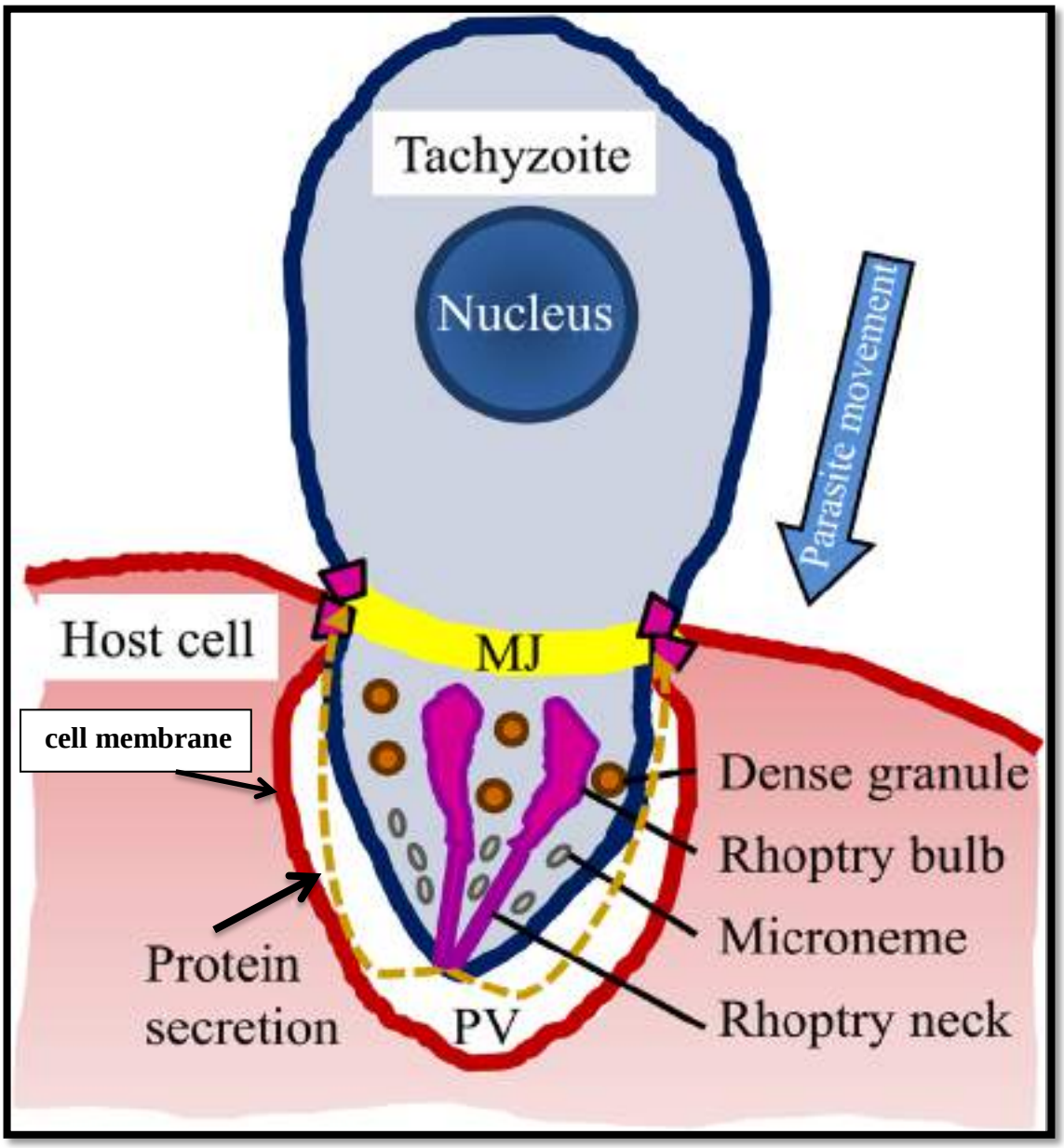

Figure 2-10: The penetration mechanism and the Parasitophorous vacuole (PV) formation of tachyzoite (Kato, 2018). MJ = Moving Junction

The tachyzoite is capable of evading the immune barrier by some mechanisms, firstly, passing cells via paracellular crossing with the unique gliding movement of the apicomplexan parasites, secondly, the process of penetration occurs when the endothelial cells are infected

by the parasite and then replicated within and released to the basolateral side, and thirdly, the process of penetration is the "Trojan horse" embodied by the transport of tachyzoite secretly in the immune cell (Soldati and Meissner, 2004; Mendez and Koshy, 2017).

At the histological level, when the parasite encounters the body's immune cell, such as the macrophages which devour it (phagocyted it), instead of eliminating the parasite, it can avoid it's grip, so this *T. gondii* stage capable to protect itself by forming PV and taking this infected cell as a safe haven from other elements of the body's immunity, here, it manipulates the macrophage, harnessing it by changing the signalling pathways (Lambert and Barragan, 2010; Lima and Lodoen, 2019).

2:5 Mode of toxoplasmosis transmission:

The disease is disseminated in poor communities with a low level of living, it showed that the intensity of infection and then the transmission is related to the community's culture and feeding habits (Sukthana, 2006; Al-Malki, 2021).

Transmission ways can be divided into two groups, (Fig. 2-11).

2:5:1 Horizontal route:

Toxoplasma gondii infected humans and other animals through one of the main transmission stages like mature oocysts, tachyzoites, and bradyzoites (Dubey *et al.*, 1998). The oocysts are excreted with cat feces, oocyst was resistant to environment factors, it contaminated any material such as soil, vegetables, fruit, water, and air with dust (Innes, 2010; Alsaady *et al.*, 2021).

Water is one of the important routes in mass transmission in the world such as occurred in Colombia, when an oocyst exists in water supplies it may be caused mass infection in that consumer area (Hernandez-Cortazar *et al*., 2017).

Soil is considered the natural source for harboring cat feces and the same time *Toxoplasma* oocysts (de Oliveira *et al.*, 2021). Earthworms act as catalysts for soil stirring thereby enhancing the possibility of infection (Bettiol *et al.*, 2000). The presence of the oocysts in places devoid of cats may be due to environmental factors such as rain washing (Gotteland *et al*., 2014). Even vegetarians contracted toxoplasmosis despite their abstinence from eating meat, but the contamination of vegetables by fecal felines was the source of transmission (Innes, 2010). Human may be acquired the infection with tachyzoite through raw cow's milk which is considered a potential source of infection (Koethe *et al*.,2017) or through blood transfusions from infected **(**Sadooghian *et al*., 2017; Mijbel, 2022). Undercooked and raw meat and unwashed kitchen tools after cutting meat are major sources of toxoplasmosis infection (Leroy and Hadjichristodoulou, 2005; Jones and Dubey, 2012; Mijbel, 2022). It can be transmitted also through organ transplantation, direct exposure to dirt, and laboratory accidents (Ayaz *et al.*, 2016; Jones *et al.*, 2016). *T. gondii* may be transmitted through insect feces or mechanical transmission on the body like a dung scarab beetle, cockroaches, and flies (Saitoh and Itagaki, 1990). *T. gondii* may be transmitted by sexual contact, and in acute infection, it can be shown in the semen (Ayaz *et al.*, 2016; Tong *et al.*, 2023).

2:5:2 Vertical route:

This transmission occurs only from the mother to her fetus, producing an infection called congenital toxoplasmosis (Kodjikian *et al*., 2004).

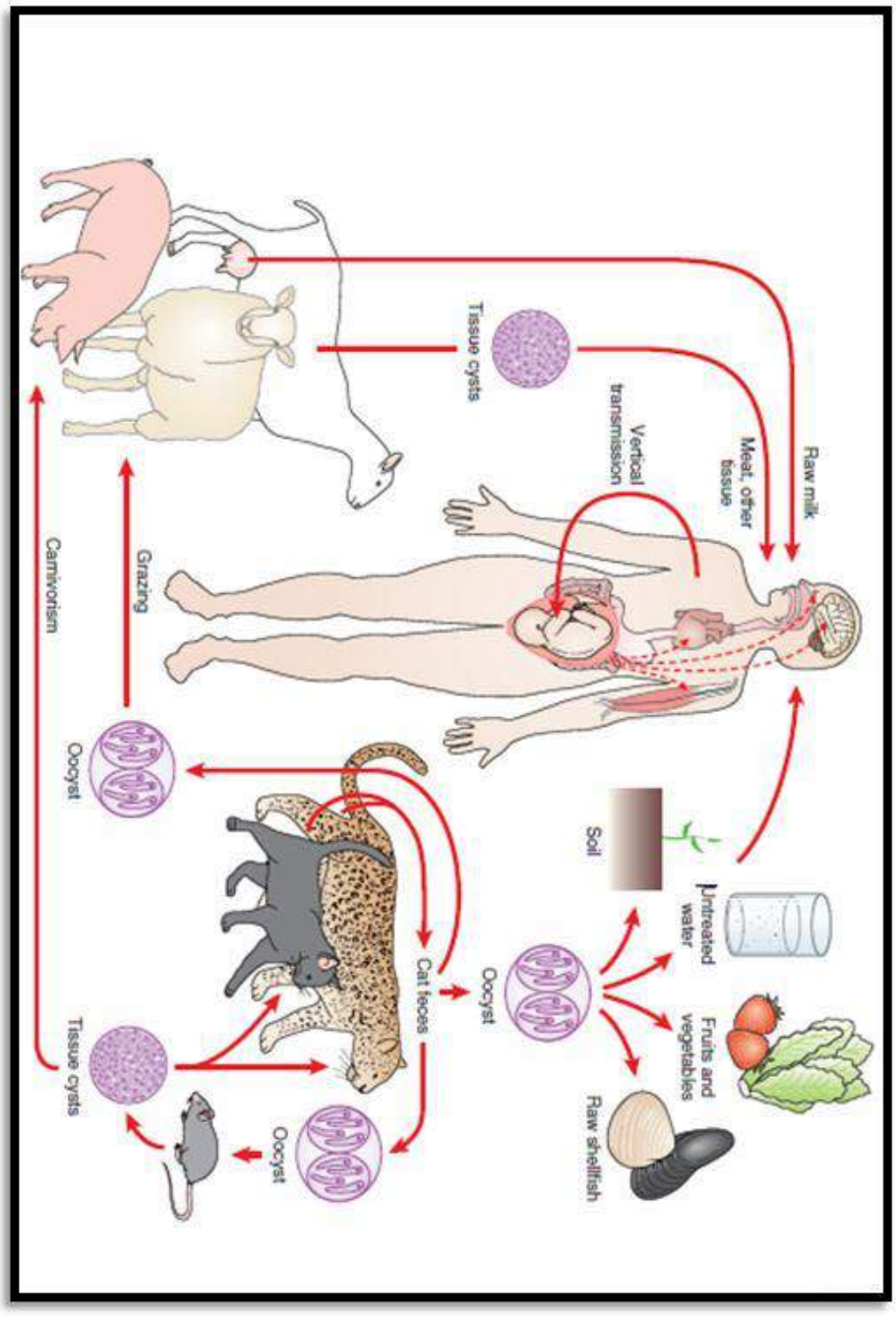

Figure 2-11: Mode of toxoplasmosis transmission (Montoya and Remington, 2003).

2:6 Toxoplasmosis Pathogenicity:

Toxoplasmosis is a disease with varied clinical symptoms (Frenkel, 1985). So in this context infection is divided as following:

2:6:1 Congenital Infection:

Congenital toxoplasmosis (CT) is a disease that occured when the parasite passes the mother's placenta to her fetus and causes a disorder intrauterine but in rare proportions, interestingly the possibility of transmission is directly proportional to the progress of gestational age and inversely with the severity of the infection, thereby the transmission rate of the disease is 15% in the first trimester of pregnancy, while it reaches to 65% in the third trimester (Paquet *et al*., 2013; Sastry and Bhat, 2018).

The infection is dangerous if the pregnant mother is exposed for the first time at the beginning of the gestational age, and it is possibly led to abortion or stillborn (Rorman *et al*., 2006).

It is worth noting that not all infected mothers transmit the infection to their fetus if the exposure was before pregnancy unless there is an immunocompromised patient (Gomella *et al*., 2004).

The severity of congenital toxoplasmosis in newborns ranges from death within days of birth to asymptomatic this disease may cause premature birth to a fetus with an enlargement liver and microcephaly or macrocephaly, Learning difficulty, mental retardation, retinitis, hepatosplenomegaly, umbilical bleeding, and jaundice (Tesini, 2020; Nagalo *et al.,* 2022).

The infant may be born with the disease, but it is subclinical, in this case, the chance of ocular toxoplasmosis is more probability (Wallon and Peyron, 2018).

2:6:2 Toxoplasmosis in immunocompetent individuals:

The people who are infected with *T. gondii* had an asymptomatic disease and remain dormant or exhibited mild symptoms that do not warn of the dangers of true *Toxoplasma* (Frenkel, 1988). In some cases, the lung is infected and symptoms coughing and shortness of breath appear, but it is not fatal for those with high immunity (Pomeroy and Filice, 1992).

The harm of *T. gondii* was not limited to the organs of the body , also caused psychological diseases by directly infecting the brain and impeding its growth, which leads to schizophrenia and other mental disorders (Torrey and Yolken, 2003; Fuglewicz *et al.,* 2017). It can cause depression and suicide due to neuroinflammation caused by the inactive parasite where it is in the brain (Kamal *et al.,* 2022). Many studies reported that *T. gondii* can cause behavioral changes (Webster *et al.,* 2013), nervousness mood and manipulated host behavior (Hugbes, 2013; mijbel, 2022), more aggressive (Berdoy *et al.,* 2002), schizophrenia (AlArdi, 2021). The degree of behavior disease is related to the site of brain damage and the *T. gondii* genotype (Lindora *et al.,* 2012).

When the host's immunity weakens, the cysts reactivate and sometimes cause myocarditis, encephalitis or even death (Cottrell, 1986; Matowicka-Karna and Kemona, 2014; Sepúlveda-Arias *et al.,* 2014).

2:6:3 Ocular Toxoplasmosis:

Ocular toxoplasmosis (OT) occurs after long time of infection with this parasite, as retinitis appears in the form of blurry vision (Tabbara, 1990; Smith *et al*., 2021). Currently, parasitologists have proven that toxoplasmosis has decreased by 50% in the world, but it attacks old age individuals (Garweg, 2016).

2:6:4:Reactivated Toxoplasmosis:

Reactivation means that the person is chronically infected with toxoplasmosis and the parasite is dormant in his tissues and opportunistically waits for Immunodeficiency, thereafter turning into the active stage (Montoya and Liesenfeld, 2004). It is represented by fatal encephalitis, accompanied by clinical symptoms such as headache, stiff neck, burdensome, memory loss, and hemiparesis (Luft and Remington, 1992). A decline in the activity of cytotoxic T-lymphocytes, a decrease in the production of cytokines, and a deficit of $CD4^{+}$ are the factors that lead to reactivating the infection (Basavaraju, 2016). The process of inactivity is interpreted as an adaptation by *T. gondii* to complement the approach to infecting another host (Frenkel, 1988). As for the recipients of the organs, symptoms appear approximately after two months, the parasite spreads in most parts of the body, and the most prominent symptoms are encephalitis and pneumonitis (Derouin *et al.*, 2008).

2:7 Genotype of *Toxoplasma gondii:*

There are more than 200 genotypes of Toxoplasma, but there is a dominant genotypes such as I, II and III, it is important to study the genotypes because it relates to a clinical variance of toxoplasmosis (Xiao and Yolken, 2015; Costa *et al.,* 2022). Where the dominant genotype was recorded in Europe is genotype II and it is often associated with congenital toxoplasmosis (Ajzenberg *et al.,* 2002; Fernández-Escobar *et al.,* 2022), genotypes II and III dominant in Africa and genotype I in Asia (Shwab *et al.,* 2014), and may the genotype I is responsible for ocular toxoplasmosis (Vallochi *et al.,* 2005).

The most successful method for distinguishing the genotypes is PCR-RFLP it was first used by Sibley and colleagues (Sibley *et al.,* 1992), and then developed by Su and colleagues (Su *et al.,* 2006). It has high accuracy in distinguishing between strains of the parasite (Ferreira *et al.,* 2008).

2:8 Laboratory Diagnosis:

Laboratory Diagnosis can divide into two ways, first way is the direct way, which is represented by PCR, isolation of the parasite, and histology, and it is used by suspected patients, and the second way is the indirect way which includes the serological tests that is used as a precautionary test in immunocompetent people and pregnant women (Jacquier *et al.,* 1995; Calderaro *et al*., 2009).

2:8:1 Serological Test:

The serological tests are dependent on one or more immune response elements such as antibodies, antigens, interleukine, cytokines, or other (Arnold and Chung, 2018). The first *T. gondii* immunoglobulin appears is IgM, it is the first type produced by the immune system as a short-term response to the pathogen that infected the body for the first time, it decreases slowly or may last for weeks or years (Teimouri *et al.,* 2020; Vargas-Villavicencio *et al.,* 2022). Two weeks after infection, IgG will appear and can remain for an extremely long period (Jones and Nichols, 2017). There are many serological tests are developed by workers in this field, and the following are some of them.

2:8:1:1 Sabin-Feldman Dye Test (SFDT):

It is one of the long time used serological tests, used to detect the *Toxoplasma* parasite, it was developed by Sabine and Feldman in 1948 (Reiter-Owona *et al.,* 1999). This test consists of the serum of the cases plus complement protein, the tachyzoite stages of the parasite, and the methylene blue stain (Pangalos *et al*., 1956).

The test result is positive if the tachyzoites cytoplasm becomes colorless, because the antibody with the complement attacks the Tachyzoite membrane and makes pores in its cell membrane, this makes the dye exit through these pores and does not settle in the cytoplasm, and the negative result if the parasite's cytoplasm becomes blue because there are no antibodies in the patient's serum (Vikrant *et al*., 2013; de Vries and Volpe, 2018).

The advantages of this test are the validity of the results, and therefore it has been adopted for a long time, and the disadvantages come from the risk of dealing with an alive parasite which may be caused infection with toxoplasmosis and another disadvantage is the test detects only IgG, which means that is not suitable to identify if the infection is acute or chronic (Vikrant *et al*., 2013; Shashtry and Bhat, 2014).

2:8:1:2 Latex Agglutination Test (LAT):

The latex agglutination test was first used by Bozdech and Jira in 1961 and many modifications were carried out on it later (Kwantes *et al*., 1972). The test includes latex particles covered with *T. gondii* antigen, and when mixed with the serum, the anti-toxoplasma antibody of positive sera binds with antigen covered latex particles and then the agglutination had been occurred, and did not occur with negative sera, it is simple in use, does not require prior training, and does not have across with other pathogens (Dubey, 2016; Sukthana *et al.,* 2001). But it is not able to differentiate between acute and chronic infections (Saadatnia and Golkar, 2012).

2:8:1:3 Enzyme-linked immunosorbent assay (ELISA):

Enzyme-linked Immunosorbent assay technique is one of the most important serological methods used in many fields to detect pathogens the antigens or antibodies of all immunogens such as toxins, the first to develop this technique was Peter Perlmann and Eva Engvall in 1960, and used since the 70^{th} of the last century (Lequin, 2005).

This technique is widely used in the world for testing serum, plasma, tissue, urine, stool, or saliva sample, the ability to diagnose a large number of samples (each microtitter plate had 96 wells) and it takes almost a short time, high sensitive and eco-friendly (Phan, 2002; Sakamoto *et al*., 2018; Moore, 2020). At this time, there are many ways to ELISA (Aydin, 2015).

2:8:1:4 Lateral Flow Immunoassay (LFIA):

Lateral Flow Immunoassay was found in old evidence that was used in a primitive way by the Egyptian a thousand years ago to know the pregnancy and the type of fetus (O'Farrell, 2009). Then it was developed in the eighties for the diagnosis of some pathogens (Fig.2-12) (Boehringer and O'Farrell, 2021). It was widely used because of it is an easy, simple design, small compact size, the possibility of interpreting the results and it is a rapid test with immediate results within minutes, with the possibility of testing even whole blood (Wong and Tse, 2008; Koczula and Gallotta, 2016).

The most prominent problems related to this test are the possibility of using only liquid samples, which has a limited range of sensitivity (Bishop *et al.*, 2019; Jiang and Lillehoj, 2021).

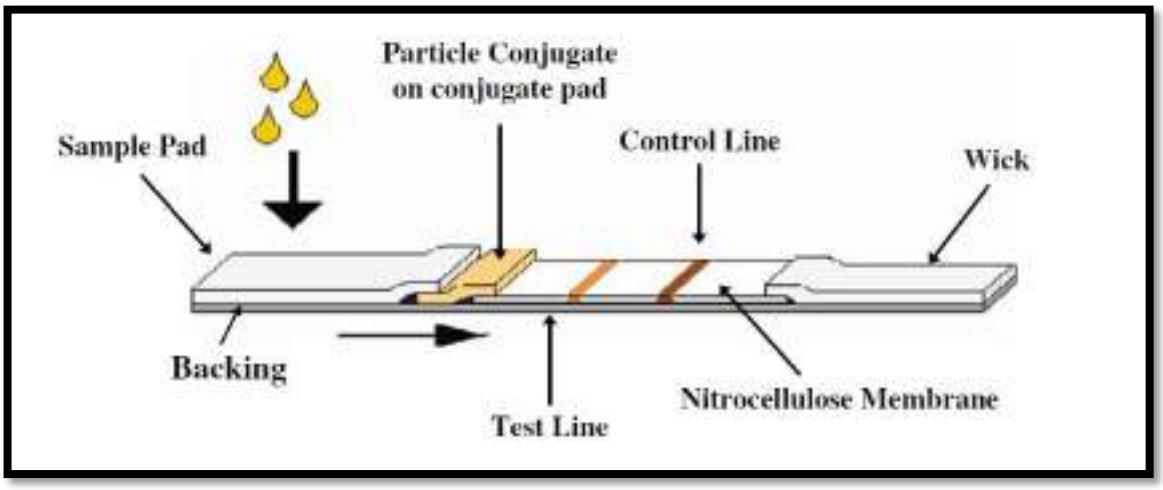

Figure 2-12: Digram showing the design of lateral flow immunoassay cassette (Wong and Tse, 2008).

2:8:1:4 Indirect Haemagglutination assay (IHA):

Indirect haemagglutination assay (IHA) was used for the first by scientist George Hirst in the year 1941 (Goryacheva, 2016). The test detects antibodies of the parasite in the patient's serum by mixing it with red blood cells, and when haemagglutination deposition occurs, the result is positive (Ybañez *et al.*, 2020). It is useful in detecting infection in epidemic cases, but it does not detect congenital and acute infections (Caruana,1980; Dubey, 2016).

2:8:2 Molecular diagnosis:

The molecular techniques used to investigate nucleic acid (DNA or RNA), and polymerase chain reactions, depends on amplifying a piece of parasite DNA into millions of copies until it is detected and used to diagnose pathogens and toxonomic organisms (Canene-Adams, 2013). Recently it was developed many types of PCR such as real-time PCR, and RFLP-PCR, can be used for detecting acute and chronic infections with highly specific analysis, as well as, it used for identifying the

pathogen's genotype (Bastien, 2002; Singh *et al.*, 2014; Wyrosdick and Schaefer, 2015).

2:8:3 Histologic diagnosis:

2:8:3:1 Immunohistochemistry (IHC):

Immunohistochemistry (IHC) is a technique that employs antibodies to detect the parasite antigens found inside the cells of suspicious tissue's for this it is capable to discover the distribution of the parasite in infected cells (Pal, 2022).

This technique was first used in 1941 by coons and colleagues (Coons *et al.,* 1941). It has high sensitivity and specificity, especially after the development of computerized technology, so it is used as a diagnostic and research test (Sukswai and Khoury, 2019; Dimenstein, 2021).

In brief, the principle of this technique involves preparing the tissue to be examined, through many protocols, then dealing with the first antibody that binds directly to the target antigen in the tissue, then adding the second antibody that contains a labelled enzyme or fluorochrome, then staining, and then photograph the result (Buchwalow and Böcker, 2010).

2:8:3:2 Hematoxylin & Eosin (H&E):

Hematoxylin and eosin are very famous dye in histopathology, as it enables us to see cellular components, Hematoxylin was first extracted in 1502 from Logwood, the tree *Haematoxylum campechianum*, which was discovered by the Spaniards, It stains the nuclei in blue and its gradations, Whilst eosin is an industrial dye that was formulated by

the director of a German chemical company in 1874, it has a red color and stains the cytoplasm of the cell (Titford, 2005; Kay, 2015; Suvarna *et al*., 2018).

The actual dye consists of hematin, which is produced by the oxidation of hematoxylin and eosin Y, the dye is a low cost and reliable results, and its working principle is based on the attraction between the dye charges and cell organelles (Feldman and Wolfe, 2014; O'Dowd, 2019).

2:9 Prevention:

As they say " An ounce of prevention is worth a pound of cure " Raising awareness reduces the chances of infection with toxoplasmosis, washing and sterilizing hands permanently after working on the farm, and washing vegetables before eating them (Lydyard *et al.*, 2021). The parasite can be eliminated in one or more of the following ways :

1) Exposing the meat at 70ºC temperature enough to destroy the oocyst and 67°C to dispose of tissue cysts (Ito *et al*., 1975; Dubey, 1996).

2) Storing the meat at -20°C for more than two days and two months at 4°C, is enough precaution against *T. gondii* (Djurković-Djaković and Milenković, 2000).

3) Treating the water with UV rays, can be eradicated *T. gondii* and avoid using ozone or bleach, as they are not effective at all (Wainwright *et al*., 2007; Dumètre *et al.*, 2008).

4) Domestic cats should be given canned and dry food and should be treated with suitable vaccines (Frenkel and Dubey, 1972; Zhang *et al.*,2013).
5) Pregnant women should not clean litter boxes (Kravetz and Federman, 2005). When cutting meat, avoid contact with the eyes and mouth, or taste food during cooking (Hill and Dubey, 2002; Wilson *et al.*, 2015).

2:10 Treatment:

The most common treatment for acute toxoplasmosis are pyrimethamine (PYR) and sulfadiazine (SDZ) combined plus folinic acid (Montoya and Liesenfeld, 2004; Dunay *et al.*, 2018). It is not preferred the use this drug in the first months of pregnancy because of it is the teratogenic effect (Chang, 1996). But it is an effective treatment to eliminate tachyzoite and save the fetus from congenital toxoplasmosis even with the presence of side effects (Schaechter, 2009).

Therefore, in the case of confirmed infection, spiramycin is given for the first third, after which it follows (PYR-SDZ) plus folinic acid for one month (Hotop *et al.*, 2012).

Recently, pyrimethamine-sulfadoxine (Fansidar) has been used to treat congenital infections of newborns for two years with folinic acid (Villena *et al.*, 1998; Petersen and Schmidt, 2003). Spiramycin is used to protect the fetus from infection through the placenta, as the drug is safe for pregnant women (Antczak *et al.*, 2016).

For AIDS patients, anti-toxoplasmosis medications must be taken for life, the most famous of which is PYR plus sulfonamide with folic acid to prevent PYR from inhibiting the bone marrow, also the combined azithromycin and clindamycin can treat toxoplasmosis AIDS patients (Nath and Sinai, 2003; Mohammadi *et al.*, 2021).

Medicinal herbs are involved in parasite treatment, as they have fewer side effects than chemical drugs (Cheraghipour *et al.*, 2021).

But there is no medicine against bradyzoite, and if the aforementioned medicines are given, they are either for prevention or to eliminate the acute infection (Lapinskas and Ben-Harari, 2019; Mayoral *et al.*, 2020).

2:11 Placenta:

The placenta is a discoid organ that formed exclusively in mammals during the pregnancy process and provides the fetus with nutrients, allows gas exchange, and eliminates waste (Benirschke *et al.*, 2006; Anderson, 2012; Burton and Jauniaux, 2015).

When the zygote is implanted after undergoing many divisions in the wall of the uterus, where this process is undertaken by cells called the trophoblast they are divided into cytotrophoblast which after implantation in the placenta, it will turn into extravillous trophoblasts (EVT) and syncytiotrophoblast (SYN) (Fig. 2-13), and the implantation site in the uterus is called the decidua, which is divided into three parts: the decidua basalis, which is located between the fetus and myometrium, decidua capsularis, and decidua parietalis, the placenta is formed by the cooperation of two surfaces, the fetal surface

(trophoblast) and the maternal surface (decidua basalis) (DaSilva-Arnold *et al.*, 2015; Mescher, 2016; Hoo *et al.*, 2020).

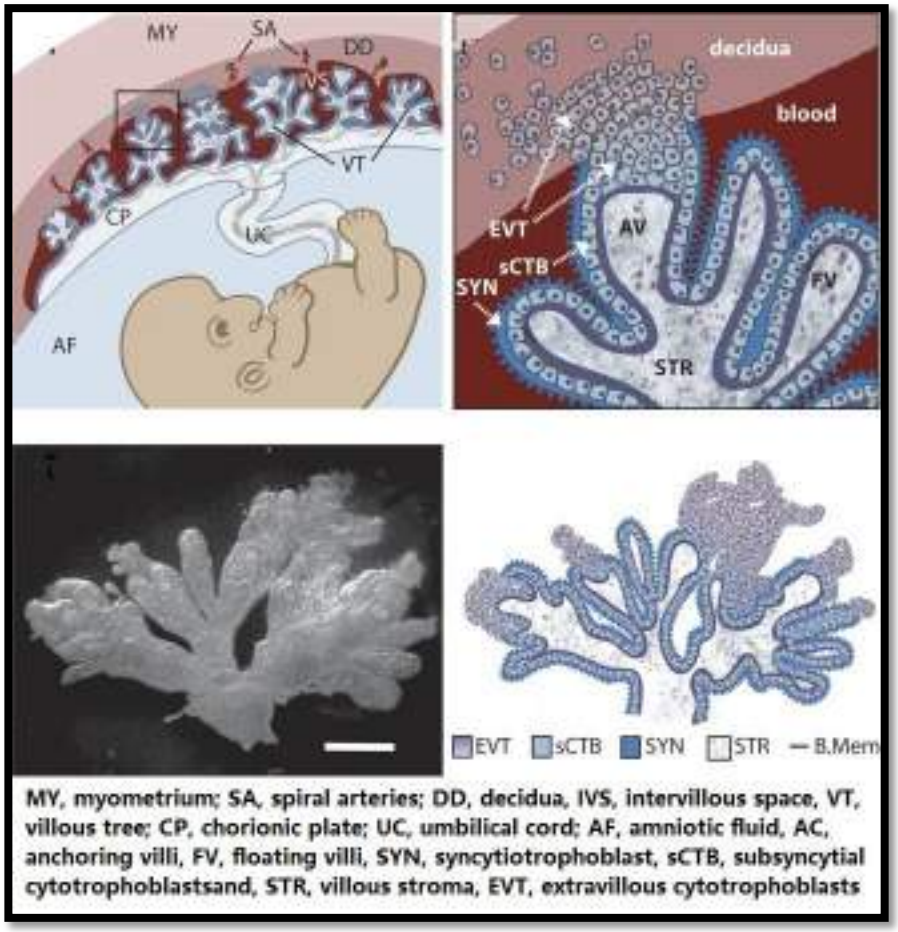

Figure 2-13: Placenta structure (Zeldovich and Bakardjiev, 2012).

The placenta is an organ that mainly participates in the health of the mother and the fetus, as any defect that occurs in structure or function affects the growth of the fetus (Brosens *et al.*, 2011; Guttmacher *et al.*, 2014). Furthermore, The placenta expresses the antigens of the paternal, so it provides for itself and the developing fetus a type of immune tolerance, which is embodied by containing neurokinin B on phosphocholine molecules which aid the fetus to not rejected from the mental immune system because it is foreign body to the mother (Guleria and Sayegh, 2007; Phil, 2010).

On the surface of the fetus, there are two types of villi, the first is floating facing the intervillous space to perform the exchange, and the second is stem villi attached to the endometrium to stabilize pregnancy in the early weeks, and the villous covered by a trophoblast (Bischof and Irminger-Finger, 2005; Vaillancourt *et al.*, 2009). Blood with nutrients is transported to the fetus through the umbilical cord, which consists of two arteries and a vein (Ernst, 2011).

The intervillous space represents an immune barrier, in this context when the parasite infects the human being, it spreads through the blood to all parts of the body (parasitemia), unfortunately, if the host is a pregnant woman, it breaches this barrier, thereby resulting in a silent miscarriage or congenital infection (Lamb, 2012; Espino *et al*., 2021). In rare probability, *T. gondii* can pass through the decidua to the fetus (Robbins *et al*., 2012).

Interesting that the placenta is considered an endocrine gland, and secreted many hormones such as Human Chorionic Gonadotrophin (HCG) which maintains progesterone levels, and Human Placental Lactogen (HPL) to Metabolic adjustment (Donnelly and Campling, 2014; Handwerger and Freemark, 2000). The placenta was the area of communication between the fetus and his mother, through which the mother can provide the fetus with all means of living and survival parameters that promote his development and growth, but when some pathogens are able to invade this area and established for whatever reason, there is no doubt, pathogens will cause an imbalance in the existing balance equation, and this depends on the species, strain and intensity of the invading organism (Ander *et al.*, 2019).

Chapter Three

Materials

&

Methods

3. Materials and Methods:

3:1 Materials:

3:1:1: Equipment and Instruments:

In the current study, many instruments, equipment and laboratory materials were used, which are summarized with the manufacturers in the following tables (3-1, 3-2).

Table 3-1: The equipment and Instruments used in this study.

No.	Equipment/Instruments	Company	Origin
1	Pipettes	Darwell	China
2	Kern PFB balance	Kern & Sohn	Germany
3	UVP	Analytik Jena	UK
4	Optimus 96G Thermal Cycler	QLS	UK
5	Stratagene Real-Time thermal cycler MX3005P	Stratagene	USA
6	Electrophoresis system	Cleaver	UK
7	Kern PFB balance	Kern & Sohn	Germany
8	Microfuge IB Centrifuge	Beckman Coulter	Germany
9	Surgical gloves	Conex	Malaysia
10	EDTA tube	Sun-vacue	Jordan
11	Gel & clot activator tube	Arth Al.Rafidain	Iraq
12	Plastic sample cup	Qingdao Jindian	China

13	Disposable syringe	Set Medikal	Turkey
14	Disposable Face Mask	Albatross	USA
15	Safety Glasses	Yl	China
16	Surgical Gowen	Henan Lantian	China
17	Scalpel handle	henry schein	USA
18	Surgical Scissors	Germanos Medicals	Greece
19	Surgical Blades	Demotek	Turkey
20	Surgical Forceps	henry schein	USA
21	Beaker	Simax	Czech republic
22	Water bath	FALC BI	Italy
23	Positively charged glass slides (Electro-Statically Charged)	SANTA CRUZ BIOTECHNOLOGY	USA
24	Hot plate	K&K	Korea
25	Immunohistochemistry Pens	Gene tech	China
26	Automated tissue processor	Histo-Line	Italy
27	Semiautomatic microtome		
28	Tissue embedding system	HESTION	China
29	Eppendorf Tubes	Sigma aldrich	USA
30	Vortex mixer	Bioevopeak	China
31	Incubater	ESCO	

3:1:2 Laboratory kits and Chemical solutions:

Table 3-2: The Laboratory kits and Chemical solutions that are used in this study.

No.	Material and Solutions	Cat. No.	Company	Origin
1	AddPrep Genomic DNA Extraction Kit	10023	Addbio	South Korea
2	OnSite®Toxo IgG/IgM Combo Rapid Test	R0234C	CTKbiotech	USA
3	RedSafe nucleic acid staining solution	21141	Intronbio	South Korea
4	Agarose LE	32034-50		
5	Safe-Green 100bp Opti-DNA Marker	G473	ABM	Canada
6	Primers	-----	Macrogen	South Korea
7	10X TBE (Tris-borate EDTA) buffer	-----	Promega	USA
8	GoTaq® Probe qPCR	A6101		
9	GoTaq® G2 Green Master Mix	M7822		
10	Mayer hematoxylin	05-06002/L	Bio-Optica	Italy
11	Harris's hematoxylin	-----	Histo line	Italy
12	Acid Alcohol Solution	-----	Sigma Aldrich	USA
13	Ammonia Water Solution	-----		
14	Absolute ethanol	-----		
15	Formaldehyde solution 37%	-----		

3:1:3: Primers that are used in this study:

3:1:3:1 RT-qPCR primers and probe:

Table 3-3: The primers and probe of the B1 gene of RT-qPCR.

Target gene	Primer name	Sequence	Ta (°C)	Product size	Reference
B1	F	3'-CTAGTATCGTGCGGCAATGTG-5'	56	62 bp	Kompalic-Cristo *et al*., 2007
	R	5'-GGCAGCGTCTCTTCCTCTTTT-3'			
	Probe	FAM-5'-CCACCTCGCCTCTTGG-3'-BHQ1	60		

3:1:3:2 Amplification of partial region of GRA6 gene (for genotyping of *Toxoplasma*):

Table 3-4:The primers of the GRA6 gene that is used for RFLP-PCR.

Target gene		Sequence	Ta (°C)	Product size	Reference
GRA6	F	3'-GTAGCGTGCTTGTTGGCGAC-5'	56	773 bp	Norouzi *et al*., 2016
	R	5'-TACAAGACATAGAGTGCCCC-3'			

3:1:3:3 Restriction enzyme data:

Table 3-5: The restriction enzyme that is used in RFLP PCR.

Restriction enzyme	Cut Site	Concentration	Buffer	Company	Country
MseI	TTAA	10.000 unit/ml	CutSmart	New England Biolabs (NEB)	USA

3:1:4 Extracting DNA kit components:

Table 3-6 : Extracting DNA kit components

Buffer & Material	Size
Spin column	100 ea
Lysis buffer	30 ml
Binding buffer	25 ml
Washing buffer 1	30 ml (Add Ethanol 22.5 ml)
Washing buffer 2	12 ml (Add Ethanol 48 ml)
Elution	25 ml
Proteinase K (20 mg/ml)	1.2 ml X 2 tubes

3:1:5 PCR solutions:

3:1:5:1 Preparation of PCR solutions:

Table 3-7:Preparation of PCR solutions used in this study.

Components	Concentration	Volume (50 µl)	Reference
GoTaq® G2 Green Master Mix	1X	25 µl	Norouzi *et al*., 2016
Forward primer	10 µM/µl	4 µl	
Reverse primer	10 µM/µl	4 µl	
ddH_2O	-	13 µl	
DNA	40 ng	4 µl	

3:1:5:2 Preparation of RT-qPCR solution:

Table 3-8: Preparation of RT-qPCR solutions.

Components	Concentration	Volume (20 µl)	Reference
DDW	-	5 µl	Kompalic-Cristo *et al*., 2007
GoTaq® Probe qPCR Master Mix	1X	10 µl	
Forward primer	10 µM/µl	1 µl	
Reverse primer	10 µM/µl	1 µl	
Probe 1	10 µM/µl	1 µl	
DNA	40 ng	2 µl	

3:1:6 PCR conditions:

3:1:6:1 Real time PCR:

Table 3-9: RT-qPCR conditions.

Phase	Tm (^{o}C)	Time	Cycles	Reference
Initial denaturation	95^{o}C	10 min	1X	Kompalic-Cristo *et al*., 2007
Denaturation	95^{o}C	15 sec.	40X	
Annealing	60^{o}C	1 min		
Final Extension	72^{o}C	30 sec		

3:1:6:2 conventional PCR:

Table 3-10: The conventional PCR conditions.

Phase	Tm (°C)	Time	Cycles	Reference
Initial denaturation	94°C	5 min	1X	Norouzi *et al.*, 2016
Denaturation	94°C	30 sec.	35X	
Annealing	56°C	30 sec.		
Extension	72°C	1 min		
Final extension	72°C	5 min	1X	

3:1:7 LFIA Test components:

Table 3-11: The Lateral Flow Immunoassay test components.

Material	Size	Cat. No.
cassette		---
capillary tubes	10 µL	---
Diluent buffer solution	5 mL/ container	SB-R0234

3:1:8 Immunohistochemistry:

3:1:8:1 The primary Antibody, anti-*Toxoplasma gondii*:

Table 3-12: Show the primary antibody that use in the the immunohistochemistry technique.

Name	Cat. No.	Company	Country
Polyclonal Rabbit Antibody IgG	MBS373041	Mybiosource	USA

3:1:8:2 The kit of immunohistochemistry:

Table 3-13: The kit of the immunohistochemistry technique.

Name of the kit	Cat. No.	Company	Country
Envision FLEX	K8000	Dako	Denmark

3:1:8:2:1 kit components:

Table 3-14: The immunohistochemistry kit components.

Material and buffer	Cat. No.
EnVision FLEX Antibody Diluent	K8006
EnVision FLEX Wash Buffer 20X as Tris-buffered saline (TBS) bath.	SM831
EnVision™ FLEX Target Retrieval Solution, High pH as Antigen retrieval solution	DM828
EnVision FLEX Peroxidase-Blocking Reagent	SM801
EnVision FLEX /goat anti-rabbit IgG-HRP as secondary antibody labeled to horseradish peroxidase	SM802
EnVision FLEX Substrate Buffer	SM803
EnVision FLEX DAB+ Chromogen	SM827

3:2 Methods:

3:2:1 Population of study:

The population of this study consisted of 40 women, distributed as 22 aborted women and 18 non-aborted women used as a control.

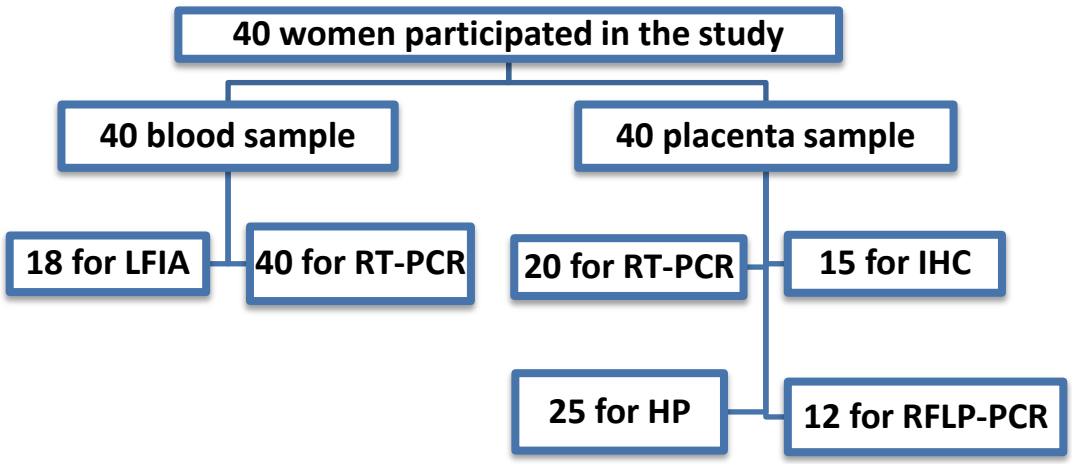

Figure 3-1 Design of Experiment.

3:2:2 The sample collection:

The sample of blood and placenta tissues were collected from participant women who visited Al-Sadr Teaching Hospital during the period from November 20, 2021, to May 19, 2022. From each participiant woman five ml of venous blood was collected and placed in an EDTA tube, and a piece of a placenta was washed well and preserved in formaldehyde 10%. Obstetric history, age, and Number of abortions are recorded for each participant women in a questionnaire (Appendix I).

3:2:3 Lateral Flow Immunoassay test (LFIA):

This test was used to detect the presence of each anti-*T. gondii* IgG and IgM in sera of the aborted women.

3:2:3:1 LFIA Principles:

When the serum is dropped into the sample hole and a sample diluent is added, the liquid flows by the action of capillary horizontally. The serum absorbted by the conjugate pad that contains

the *Toxoplasma* antigen and a control antibody both conjugated with colloidal gold. If the serum contains anti-*T. gondii* IgM or IgG, it will bind to *Toxoplasma* antigen to form an immune complex. To be captured, complexed by the pre-coated anti-human IgG on the line, which produces a red color and the intensity of the color depends on the amount of antipeptide present in the blood sample and the same case in the IgM line, and at the control line, it should become red because the absence of color indicates that the test has been damaged and must be repeated.

3:2:3:2 Procedure of LFIA test:

1- Initially, the blood samples were left in the Gel & clot activator tubes for 15 min at room temperature.

2- Centrifuging the blood sample at 3000 rpm for 10 min to separate the serum.

3- Then 10 µL of serum was taken by capillary tube and added into the cassette well.

4- Added 2 drops (approximately 60-80 µL) of the sample diluent immediately after the serum.

5- The cassette was left for 10-15 min on a flat surface where there is no strong airflow.

6- If a red line appears on IgM, this means the infection is acute positive, and if IgG means chronic positive infection, but if a red line appears only on the C (control line), it means that the sample is negative for toxoplasmosis.

3:2:4 Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR) test was used for the identification of the *T. gondii* of both blood and placental tissue.

3:2:4:1 Extracting of DNA:

The DNA of *T. gondii* was extracted from blood and placenta samples by using the AddPrep Genomic DNA Extraction Kit (Addbio, South Korea), and according to the company's instructions that attached with the kit.

3:2:4:1:1 Procedure of extracting DNA from blood:

1. 1.5 ml micro-centrifuge tube was prepared with 20 µl of Proteinase K solution (20 mg/ml).
2. 200 µl of the blood sample was added to 1.5 ml micro-centrifuge tube containing Proteinase K solution.
3. 200 µl of Binding Solution was added to the tube, and mix well for 15 sec by the vortex.
4. The mixture was incubated at 56℃ for 10 min.
5. 200 µl of absolute ethanol was added and mix well by the vortex for 15 sec.
6. With a 2.0ml collection tube, the lysate was moved into the upper reservoir of the spin column without moistening the rim.
7. Centrifuging at 13,000 rpm for 1 min, and discard the flow-through and collect the spin column with the 2.0 ml collection tube.
8. 500 µl of washing 1 solution was added to the collection tube and the spin column, and continue as in Step 7.
9. 500 µl of washing 2 solutions was added to the spin column with the collection tube, and continue as in Step 7.

10. Spin column was dried for 1 min to residual ethanol by centrifuging at 13,000 rpm.
11. The spin column was moved to a new 1.5 ml micro-centrifuge tube.
12. By the spin column with the micro-centrifuge tube added 100~ 200 µl of Elution Solution, and leave it for at least 1 min.
13. Elution the DNA was carried by centrifuging at 13,000 rpm.

3:2:4:1:2 Procedure of extracting DNA from tissue (with a specific modification for a purpose of parasitic DNA extraction):

1. Placental tissue was cut to about 20 mg, then transfers into a 1.5 ml Eppendorf tube, and added 200 µl of Lysis solution .
2. In the sample tube, was added 20 µl of Proteinase K solution (20 mg/ml), mixed by the vortex, and incubate at 56°C until the tissue is totally lysed.
3. 200 µl of Binding Solution was added to the sample tube, then mix by the vortex for 15 sec.

Then, followed steps 4 to 13 of item 3:2:4:1:1.

3:2:4:2 The preparation of primers:

According to the manufacture instructions, the primers (Initially lyophilized) were dissolved in free DDW to achieve a final concentration of 100 µM/µl, which was used as a stock solution and kept at -20^{o} C. Preparing a concentration of 10 µM/µl from the stock primers to be used as a working primer.

3:2:4:3 Electrophoresis:

Agarose gel Electrophoresis (AGE) use to determine the quality of DNA extraction and imaging the size of the DNA fragment after the end of the PCR program, the type of product affected by the concentration of the gel thus is 1-2% for normal PCR and 0.7% for DNA quality.

1. The Preparing of the Agarose gel has been conducted according to the protocol that was explained by Sambrook *et al.* (1989). which dissolved 1 gm of agarose in 100 ml of a 1X Tris-borate EDTA buffer and heated up until it boils in a microwave machine.
2. Then cooling at 45-50^{o} C then RedSafe nucleic acid staining solution was added to the gel and poured into the gel caster placed the comb at the end of the costing tray and then lefting it for about 30 min to complete the polymerization.
3. The gel plate was repositioned into the gel tank and filled with the running buffer to the point of covering the gel surface.
4. The loading of the PCR products is done by inserting 5 µl of PCR product of blood and placenta samples into the middle of the slot
5. Added 5 µl of Safe-Green 100bp Opti-DNA Marker to the first slot to perform as a marker that can via it measures the size of PCR products opportune system of electrophoresis was set at a continuous current of 90 volts for 45 min, and finally to visualize the results transfer the gel into the UVP system under a 320nm UV light.

3:2:4:4 RFLP assay:

1. From NEB, USA obtained a buffer for the DNA manipulations and Restriction endonuclease (MseI).
2. According to the suitable purposes of digests, restriction digests were prepared in a final volume of 40 µl.
3. Samples were prepared to meet the following standards for preparative and analytical purposes: with a 1X appropriate restriction buffer and 10 units of the needed restriction enzyme, 1-3 µg of PCR product was digested.
4. In the thermal cycler at 94℃ for 5 min followed by 35 cycles of 94℃ for 30 sec and 1 min at 72℃.
5. After that, the digests were incubated for 3 hours at 37°C (or enzyme-suitable temperature) and then put through electrophoresis to visualize the results.
6. In conclusion, for each tested sample, three genotypes were predicted to be obtained.

3:2:4:5 Real-Time Quantitative PCR (RT-qPCR) procedure:

1. Some samples were used in the implementation of the probe-based RT-qPCR.
2. Specific probe and one pair of specific primers were used in the amplification process of the conserved region of *Toxoplasma gondii* gene B1.
3. Use 2ng of genomic DNA and GoTaq® Probe RT-qPCR Master Mix (Promega, USA) and Reactions were performed in 0.2 µl wells, in a total 20 ml volume .

4. Then the wells positioned in the thermal cycler of RT-qPCR (Stratagene, USA) at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 s and 1 min at 60℃ .
5. The success rate of amplifying was better than 95% and 1% as the calculated error rate on PCR duplicates.

3:2:5 Histological Study:

The histopathology and IHC were performed in the U-gene laboratory in AL Najaf province

3:2:5:1 10% Formaldehyde solution preparation:

Ten ml of formaldehyde was mixed with 90 ml of tap water and then shaken well to get a 10% dilute solution used for tissue preservation at room temperature.

3:2:5:2 Procedure of Immunohistochemistry:

For the detection of *Toxoplasma gondii* in placental tissue, the Anti-*Toxoplasma gondii* primary Antibody (Polyclonal Rabbit Antibody, Mybiosource, USA) was used in the current study, it was diluted 100 folds with antibody diluent and then followed the following steps:

1- Sliced the placenta sample to 0.5 cm thickness and put them in plastic cups and use an automated tissue processor for dehydration and clearing.
2- Using a tissue embedding system to embed the placental tissue in paraffin.
3- Then cutting the paraffin-embedded placenta at 4 µm thickness by semiautomated microtome.

4- Tissue sections were placed in a water bath carefully, then by using a hot plate mounted the tissue on positively charged glass slides Charge.

5- The placenta sections were heated at 55^{o} C in an oven and then deparaffinized by two changes of xylene for 10 min each , and rehydrated by ethanol alcohol in four changes: 100%, 100%, 90% and 70% for two min each, consecutively.

6- Then the tissue sections were rinsed in distilled water and submerged for five min in a TBS buffer bath.

7- Heat the Antigen retrieval solution at 60^{o} C and incubated it in a water bath at 97^{o} C for 25 min pour it into a glass jar then placed tissue sections inside it and left at room temperature to cool for 20 min.

8- The sections were rinsed with distilled water and dipped in a TBS buffer bath for 5 min to remove the excessive buffer the tissue section and by tissue paper wiped them gently.

9- With a special wax pen draw a circle around the tissue section on the slides to confine the reagent only on the placenta tissue of the slide then rinsed the tissue section and flooded for 5 min each in two changes of a TBS buffer bath and removed the excessive buffer from tissue and wiped them softly by tissue paper.

10- The tissue sections were immersed with 100 µL of blocking reagent (peroxidase block solution) and incubated in a humidity chamber for 10 min then flushed the tissue section and flooded for 5 min each in two changes of TBS buffer bath and the step for removing excess buffer was repeated.

11- The placenta tissues were put with 100 µL of diluent Anti-*Toxoplasma gondii* primary antibody and moved in the humidity chamber to incubate at room temperature for one hour then lushed the sections and flooded in two changes of TBS buffer bath for 5 min each, the step for removing excess buffer was repeated.

12- Treating the placenta sections with 100 µL of secondary antibody labeled horseradish peroxidase (HRP) and incubated at room temperature for 30 min in a humidity chamber and flooded in two changes of TBS buffer bath for 5 min each, the step for removing excess buffer was repeated.

13- prepared DAB+ (3,3'-Diaminobenzidine) substrate-chromogen solution (by adding Chromogen to 1 ml of EnVision FLEX Substrate Buffer + one drop of EnVision FLEX DAB) and immersed the section with 100 µL of it, and incubated for ten min in the humidity chamber, then flooded in two changes of TBS buffer bath for five min each after lushing the sections.

14- After 3 min of counterstaining with Mayer hematoxylin, the tissue sections were rinsed in tap water.

15- The sections were dehydrated by passing them through three successive concentrations of ethanol 70%, 90%, and 100% for 2 min each Consecutively, then immersed in two changes of xylene for 10 min each.

16- Slides were covered with mounting media (DPX) and then with cover slips.

17- Finally, examined the tissue sections were under a light microscope at 100x and 400x magnifications.

3:2:5:3 Procedure of Histopathology:

Repeat the same steps for the IHC method 1+2+3

4- Place the placenta sections in the water bath, then by hot plate mount it on glass slides.
5- The sections were deparaffinized by two changes of xylene for 2 min each, and then rehydrated by passing them through three concentrations of ethanol 100%, 90%, and 70% for 2 min each.
6- The sections were rinsed with tap water, removed the excess water gently with tissue paper, and stained with Harris's hematoxylin for 5-8 min, then rinsed again for 2 min in tap water to remove the excess stain.
7- Then immersed the sections into 1% acid alcohol solution for 20 sec to differentiate excess hematoxylin stain before being washed in tap water for 1 min.
8- For bluing the hematoxylin stain, was immersed placenta sections in 0.2% ammonia water solution, were for 1 min and washed in running tap water for 5 min.
9- Placenta sections were rinsed in 95% alcohol for 20 sec before being counterstained for 5 min with eosin stains.
10- The sections were dehydrated by passing them through three successive concentrations of ethanol (70%, 90%, and 100%) for 2 min each, and then, cleared with two different xylene changes for 2 minutes each.
11- Finally, the placenta sections were examined by light microscope at 40x, 100x, 200x, and 400x magnifications.

3:2:6 Statistical Analysis:

The data of the results of the study are statistically analyzed using the $SPSS_{24}$ software, and by chi-square test (Griffith, 2007).

Chapter Four

Results

&

discussion

4: Results and Discussion:

4:1 Serological Test:

The result of the immunological test using the LFIA technique (Table 4-1) find that 38.9% of women were infected with toxoplasmosis, 85.7% of infections are among aborted women compared to 14.3% of *T. gondii* infection were non-aborted women, and shows that 85.7% of the infected women had at least one abortion, compared to 36.4% of the uninfected women. And it also noted that 60% of abortions were recorded among women who were infected with toxoplasmosis compared to 40% of their abortions were from infected women.

Table 4-1: Toxoplasmosis sero-infection rate among the aborted and non-aborted women using LFIA.

Anti-Toxoplasmosis		**abortion**			χ^2	***P***
		Having	**Without**	**Total**		
		No. **(%)**	No. **(%)**	No. **(%)**		
IgG seropositive	No. (%)	6 (85.7) **(60.0)**	1(14.3) **(12.5)**	7 (100) **(38.9)**	4.219	0.04
IgG seronegative	No. (%)	4 (36.4) **(40.0)**	7 (63.6) **(87.5)**	11 (100) **61.1**		
Total	No. (%)	10 (55.6) **(100)**	8 (44.4) **(100)**	18 (100) **100**		

The results of the study were in line with the finding of Muqbil and Alqubatii in Yemen (2014) and ALkanaq *et al.* (2020) in Qadisiyah, Iraq. These results is higher than that found in some other regions of the world such as Subasinghe *et al.* (2011) in Sri-lanka, Imam *et al.* (2016) in Saudi Arabia, and Paul *et al.* (2022) in Bangladesh and higher than some studies in Iraq like AL Mossa (2009) in Baghdad, Alouci (2018) in Anbar, Mohammed (2018) in Salahaldeen and Al-Masoudi *et al.* (2020) in Babylon. And less than Al-Omer and Al-Marsoomy (2021) in Nineveh.

In these results, all were positive to IgG and no anti-*Toxoplasma gondii* IgM was shown, these findings agreed with Frimpong *et al.* (2017) and Gomez *et al.* (2018). This indicated that all infections were chronic (Teimouri *et al.*, 2020).

4:2 Molecular diagnostics:

4:2:1 Real time PCR for blood:

Forty blood samples were tested by RT-qPCR technique to detect the *T. gondii* in two groups. The results did not able to detect the DNA of *T. gondii* in the blood samples.

Table 4-2: Detection of the blood samples by RT-qPCR.

Type of delivry	No. exam	Infection rate %
Normal	18	0 (0.0%)
Abortion	22	0 (0.0%)
Total	40	0 (0.0%)

These findings were closely similar to the findings of Shaker *et al*. (2018) but disagreed with Al-nasrawi *et al* (2014) who found that 16% of aborted women were positive.

The rate of infection could be explained that the parasite in the blood is rare and difficult to detect by one of the molecular tests, it is hidden in the monocytes within the PV and also the presence of tachyzoite is in a short time in the body fluids (Unno *et al.*, 2008),

4:2:2 Real time PCR for placenta:

Twenty samples of placental tissue of aborted women were subjected to RT-qPCR to detect *T. gondii* DNA, the RT-qPCR technique detected that (10%) of women are infected with toxoplasmosis (Table 4-3 and Fig. 4-1).

Table 4-3: RT-qPCR test for placental tissue samples.

No.	Positive samples (%)	Negative samples (%)
20	2 (10%)	18 (90%)

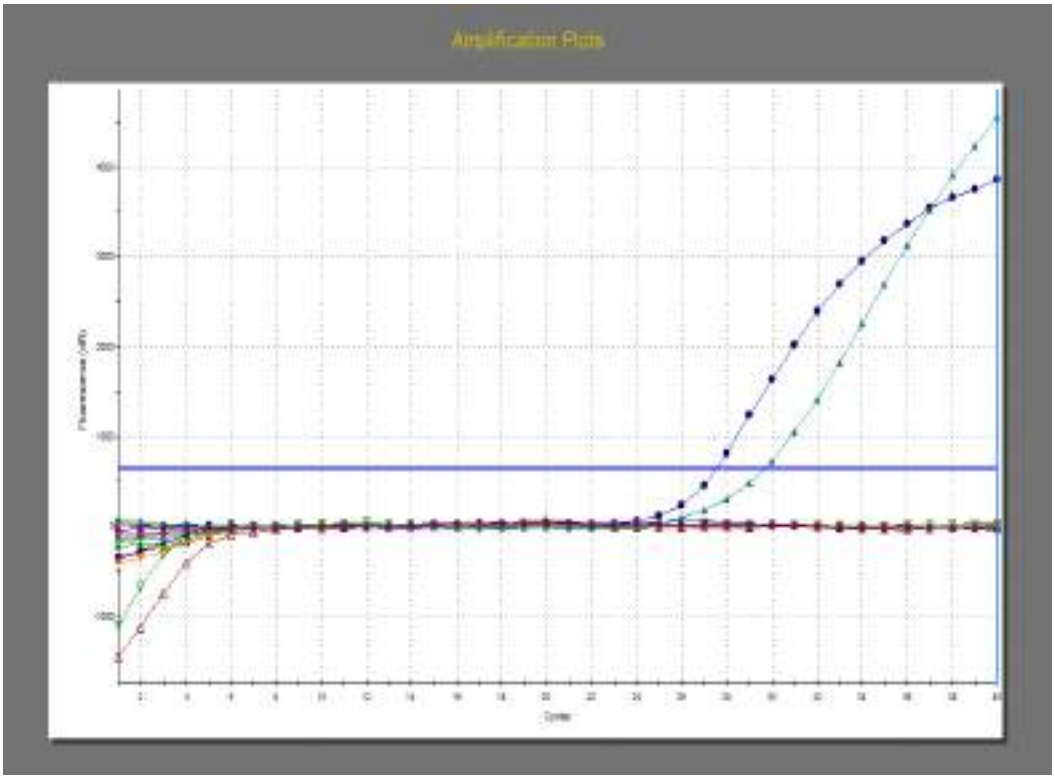

Figure 4-1: RT-qPCR analysis of the placental tissue.

The results of the current study agreed with A'aiz (2016) who showed in his study that the parasite was amplified in four placental tissues only out of 100 samples, Meixner *et al.* (2020) found only nine positive placenta tissue of 200 sample, and disagree with AL-Dujaily and Abdul-Amir (2014) who found that 50% of aborted women were infected with *Toxoplasma* by RT-qPCR technique. The low sensitivity of this technique may be due to low capability in detecting or sensing the parasite in the macrophage and in tissue cyst (Marino *et al.*, 2017).

4:2:3 Detection of *T. gondii* genotypes:

The present study revealed, using the RFLP-PCR technology, the presence of the three main genotypes of *Toxoplasma* parasite, I, II, and III in the placentas of aborted women, where the frequency of the genotype I was the most common among all the other genotypes, with

a frequency of 50%, followed by the genotype II with a frequency of 33.33%, and finally genotype III with a frequency of 16.67% (Table 4-4).

Table 4-4: Detection and genotyping of *T. gondii* of some placenta samples from aborted women by conventional PCR-RFLP assay.

Sample	**Genotype of *T. gondii***		
	Genotype I	Genotype II	Genotype III
Genotype%	50%	33.33%	16.67%

This finding is consistent with what Al-Hadraawy *et al.* (2019) found in their study in Al Najaf province I,II, and III were 60%, 25% and 15% respectively, but do not agree with the study of Mohammed *et al.* (2015) who found that the genotypes II is the dominant genotype in Baghdad province and Al-khanak and Salman (2021) in Wasit Province who found that the genotype III is dominant. In Egypt, Abdel-Hameed and Hassanein (2008) found that the genotype II is the most frequent at 87% and genotype I at 13% but they do not find genotype III. These variation in the distribution the of genotype may be due to the difference in climatic conditions and dietary habits especially if the meat was imported from other regions (Pomares *et al.*, 2011).

4:3 Immunohistochemistry Test:

4:3:1 Relationship between toxoplasmosis and the aborted in women:

Using of the Immunohistochemistry technique for the tissue sections of 15 placentas of aborted women found that 66.67% (8/12) of aborted women were infected with *T. gondii* compared to 33.3% (4/12) among non-infected. On another hand, it was recorded that 20% (2/10) of toxoplasmosis infections in women are non-aborted. And it shows that 33.33% of aborted women are non-infected with toxoplasmosis. Using the IHC technique shows a statistically significant relationship between toxoplasmosis and the abortion (X^2=13.930, p=<0.001).

Table 4-5: percentage of immunohistochemistry tests of aborted women and non-aborted women that were infected with toxoplasmosis.

Toxoplasmosis		History of abortion		Total	χ^2	p
		Yes	No			
uninfected	No.(%)	4(80) 33.3	1(20)	5	13.930	<0.001
infected	No.(%)	8(80)	2(20)	10		
Total		12(66.7)	3(33.3)	15		

4:3:2 Toxoplasmosis pathological effect on the women's placenta:

The rabbit polyclonal anti-*T. gondii* as primary antibody (Mybiosource, USA) and goat anti-rabbit IgG as the secondary antibody (EnVision FLEX, Denmark) were used to detect the localizations of *T. gondii* in various tissues of the placenta of aborted

women using the IHC technique. This study was carried out using a specific kit (Envision FLEX, Dako, K8000, Denmark) for detecting the *T. gondii* and using DAB^{+} as a staining system.

The IHC study of the placentas of aborted women revealed that *T. gondii* stages are localized with a high density in various tissues of the placenta such as trophoblastic and endothelial cells (Fig. 4-2). *T. gondii* was found also in placental villi (Fig. 4-4), and pseudocyst spreads as shown in (Fig., 4-13).

The maternal-fetal interface (MFI) tissues (Fig. 4-7) harbor a high density of *T. gondii*, this finding is also seen by Zhang *et al*. (2019). Some attributed the high density of this parasite in trophoblastic tissues to the high levels of prostaglandins in this infected site as immune responses from maternal defence against *T. gondii*, in the same field, it is reported that the increase in the level of prostaglandin is inducing the increase of the proliferation of *T. gondii* tachyzoites in Hofbauer cells of trophoblastic tissues (Barbosa *et al*., 2014).

These findings are consistent with what some studies have found about the necrosis in the trophoblastic tissue, and they attribute this phenomenon to the rise of the average of apoptosis in the toxoplasmosis infected trophoblastic tissues (Zhang *et al*., 2015).

Some observed that the infection of women with toxoplasmosis during early pregnancy leads to increase the production of chemokine receptors by the maternal immune system, which is responsible for embryo apoptosis and fetal resorption (Nishida *et al*., 2021). The decidua is distinguished by containing some immune cells like macrophages, natural killer cells, T cells, B cells, dendritic cells, and

others (Faas and Devos, 2017). In healthy pregnant women, the uterus mucosa is distinguished by immunological tolerance against fetus allergens and at the same time it protected the MFI from invading or attacking pathogens (Yang *et al*., 2019).

In MFI, the macrophages were differentiated into two phenotypes, first is classically-activated (M1) macrophages, and second, is alternatively-activated (M2) macrophages (Wang *et al*., 2022). The M2 phenotype in decidual tissues is accountable for secreting growth factors and cytokines of anti-inflammatory that boost wound curing, tissue remodelling and refine the metabolism and endocrine signaling in placenta tissue (Marray and Wynn, 2011).

These secretions form a microenvironment that acts as an immunosuppressive to brake the mother's immune response against her fetus allogeneic and then maintain the gestation (Liu *et al*., 2021). Using the IHC technique showed a high density of macrophages in the decidua of aborted women (Fig.4-3), this observation agrees with what showing by Guenther and his colleagues, who found a significant rising in the population of the decidua macrophages of aborted women, and also they confirmed that the M2 phenotype acts as maternal immunosuppressive and inhibited the inflammatory activities, and thus it contributes effectively to the maintenance and perpetuation of pregnancy, while M1 phenotype is responsible for the inflammatory activities that contribute to the acceleration of fetal abortion (Martinez *et al*., 2006 ;Guenther *et al*., 2012).

For this, the equilibrium polarization between M1 and M2 macrophage phenotypes is considered indispensable for placenta

plasticity and maintenance the gestation (Yao *et al*., 2019). And The M2 macrophages are playing a role in the in the immune-tolerance of the maternal immune system for her fetus (Wheeler *et al*., 2018).

When *T. gondii* invades the decidua, it is manipulating in the balance polarization of M1/M2 (Zhang *et al*., 2017), and the M2 phenotype was exchanged by the M1 phenotype, this leads to alter the MFI's microenvironment and activated of the inhibitors of cell proliferation which causing significant damage in placental tissue (Mills, 2012), which are caused some complication in MFI and this may lead to abortion (Guenther *et al*., 2012).

It can be concluded that there are many complications that can occur due to *Toxoplasma* infection that may contribute to pregnancy failure and abortion, but sometimes, the professional phagocytes of the innate immune system such as dendritic cells and monocytes eliminate the parasite by producing some interleukins such as IL-12 (Yarovinsky *et al*., 2005; Pifer and Yarovinsky, 2011; Koblansky *et al*., 2013; Tosh *et al*., 2016). *T. gondii* suppresses the immune tolerance that occurs naturally during pregnancy and Decidual natural killer (dNK) cell cytotoxicity is increased to cause abortion (Liu *et al.*, 2014; Sun *et al.*, 2022).

Pathological change in the tissues of the placenta for aborted women had been seen in prominent observations such as Hemorrhage in the spaces of necrotic epithelial cells (Fig.4-8, 4-9), and the *T. gondii* was found in the epithelial cells (Fig.4-2, 4-4) and congestion of the lumen of blood vessels as a result of the presence of the parasite in them (Fig.4-4), and aggregations of *T. gondii* of in sinusoids of the placenta

(Fig.4-11, 4-12). All these changes cause abortion, as a result of the parasite passing the placenta (Kadhim *et al*., 2016). And that the hemorrhage that occurs in the tissues of the placenta is due to damage to the wall of blood vessels (Ismael and Salih, 2019).

T. gondii can transmit to the placenta and causes focal lesions that either cause abortion or spread throughout the body of the fetus to settle in the central nervous system and be a congenital infection (Hill and Dubey, 2002).

One case (Fig.4-13) was of an aborted mother, induced abortion, of a fetus with severe deformity, and herein, this case was shown to be positive by only IHC, which indicated for high sensitivity of this technique to identify the infection. Repeated abortions are attributed to neglect of treatment against *Toxoplasma*, which causes the parasite to settle in the endometrium (Ali and Rashid, 2009), most of the participants in the current study had been suffering from previous abortions.

Where these observations agree with Stensvold *et al.* (2022) who found a chronic infection in the villitis of the placenta, Sousa *et al.* (2021), Al-Mussawi *et al.* (2009) who noted changes in the *T. gondii* infected tissue such as villi inflammation and necrosis in the trophoblast, Silva *et al.* (2013) which the bradyzoites were observed in the tissues of the placenta, El-Hashimi *et al*. (2014) that found the parasite in trophoblast, Khalil *et al* (2016), and Mesquita *et al.* (2019) which found Severe inflammation in the villous lining and the rustle of the current study disagrees with Rosa *et al.* (2001) who did not found *T. gondii* stages such as tachyzoites in tissues, the study finds that the infection rate by using IHC among abortion women iss

66.67%, this finding disagrees with AL-marsome *et al*. (2011) who found only 36% are infected.

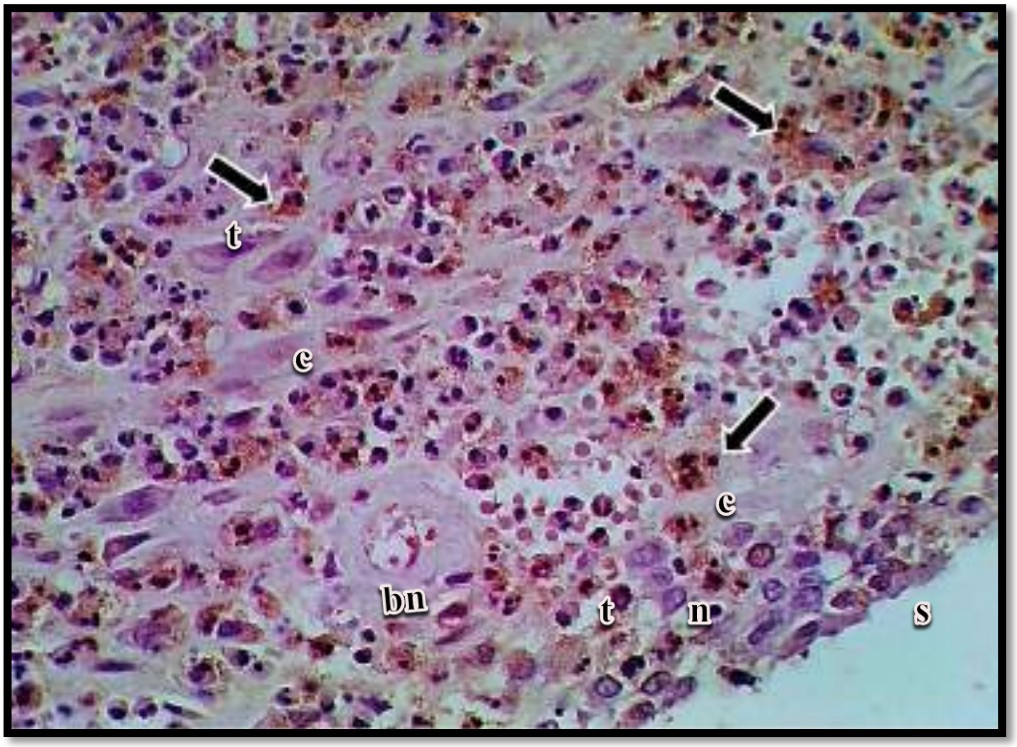

Figure 4-2:Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) indicated the presence of *T. gondii* within epithelial cells of the placenta. Hematoxylin and DAB by IHC. (40X10)X. t=tachyzoite, c=pseudocyst, n=necrosis, bn=blood vessels, s=stroma

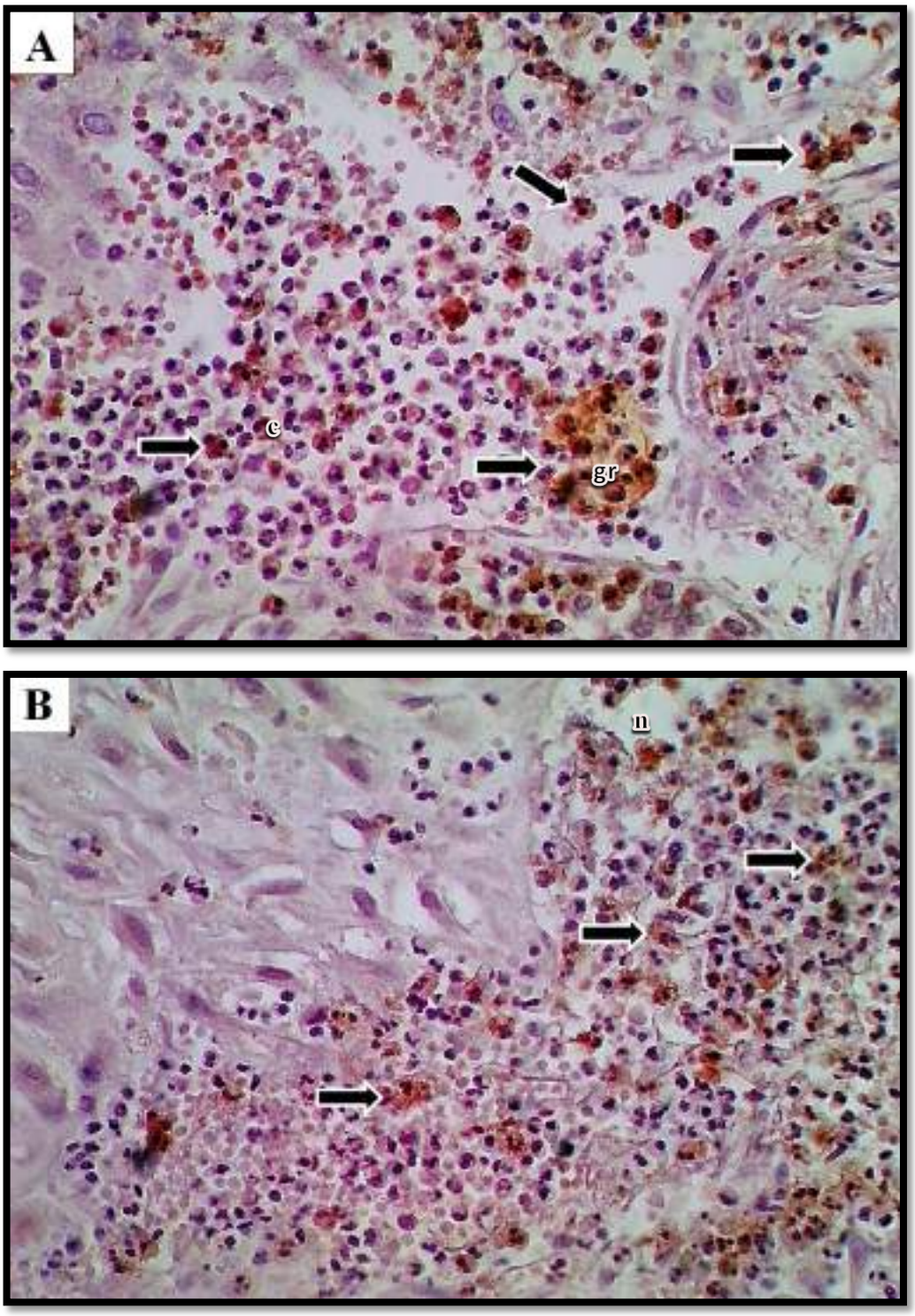

Figure 4-3: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) indicated the presence of *T. gondii* within hemorrhage that occupied the spaces of necrotic epithelial cells of the placenta and the high density of the parasite is clear. Hematoxylin and DAB by IHC. A and B (40X10)X
c=pseudocyst, n=necrosis, f=fibrosis in stroma, gr=aggregation of *T. gondii* tachyzoite.

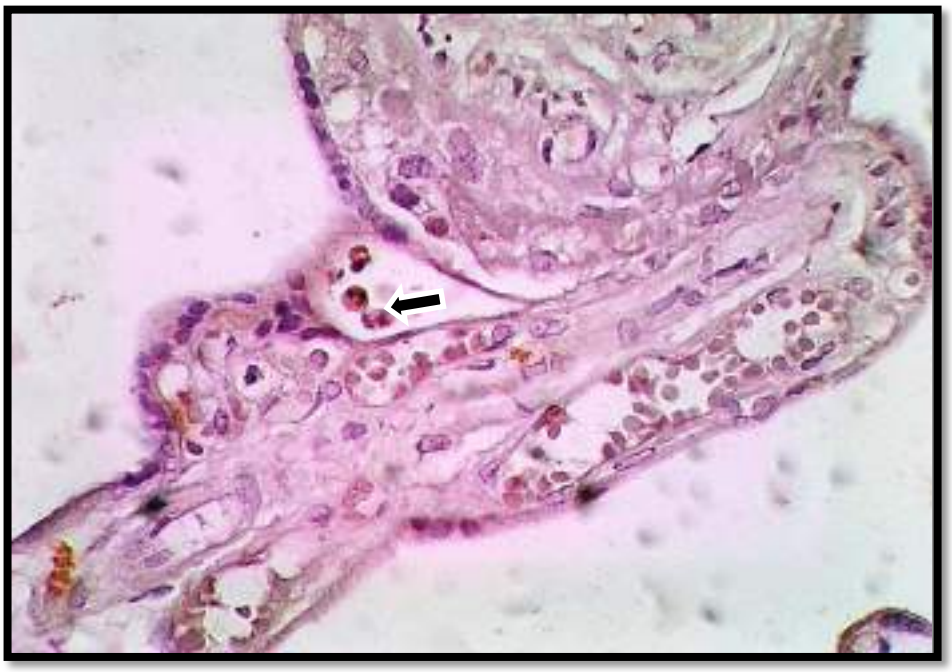

Figure 4-4: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) indicated the presence of *T. gondii* within epithelial cells in placental villi and in the lumen of blood vessels of the placenta. Hematoxylin and DAB by IHC. 40X10)X. t=tachyzoite, c=pseudocyst, d=degradation

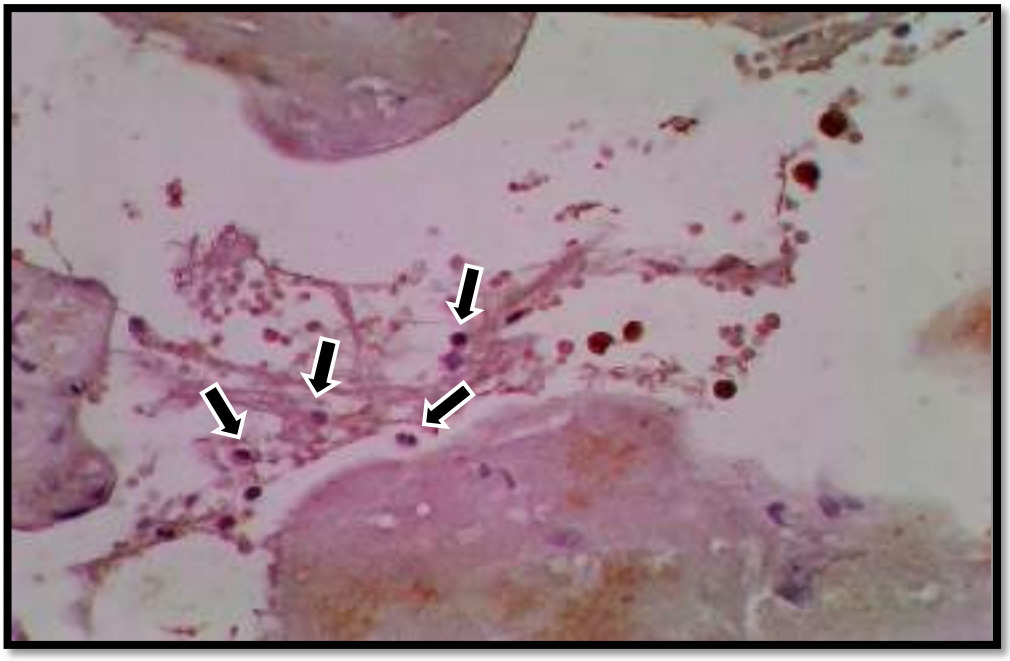

Figure 4-5: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) indicated the presence of *T. gondii* in the intervillous space of the placenta. Hematoxylin and DAB. (40X10)X.

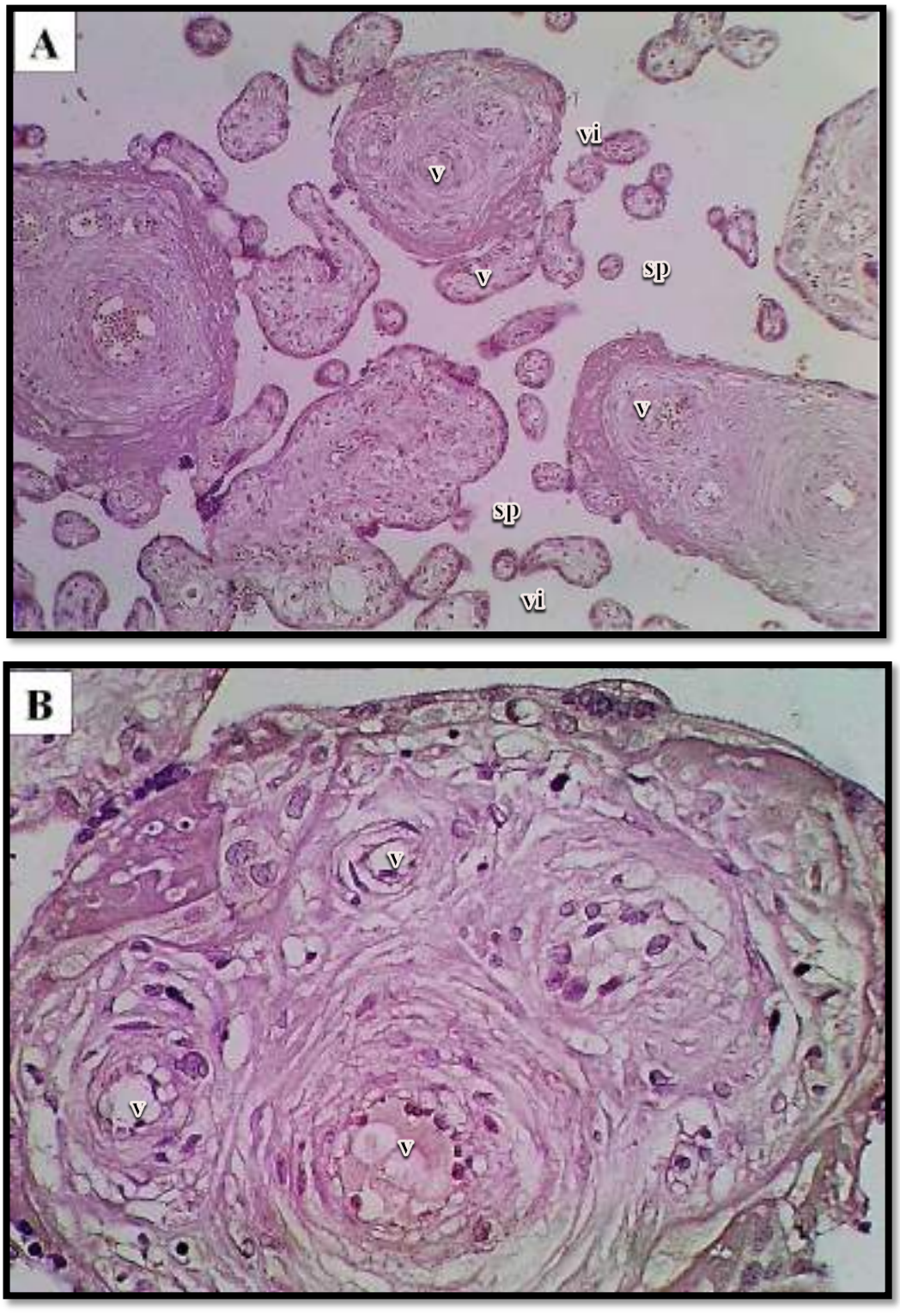

Figure 4-6: No expression of anti-*Toxoplasma gondii* primary antibody in the placenta. Hematoxylin and DAB by IHC. A: (10X10) and B: (40X10)X. v=vessel, vi=villi, sp= intervillous space.

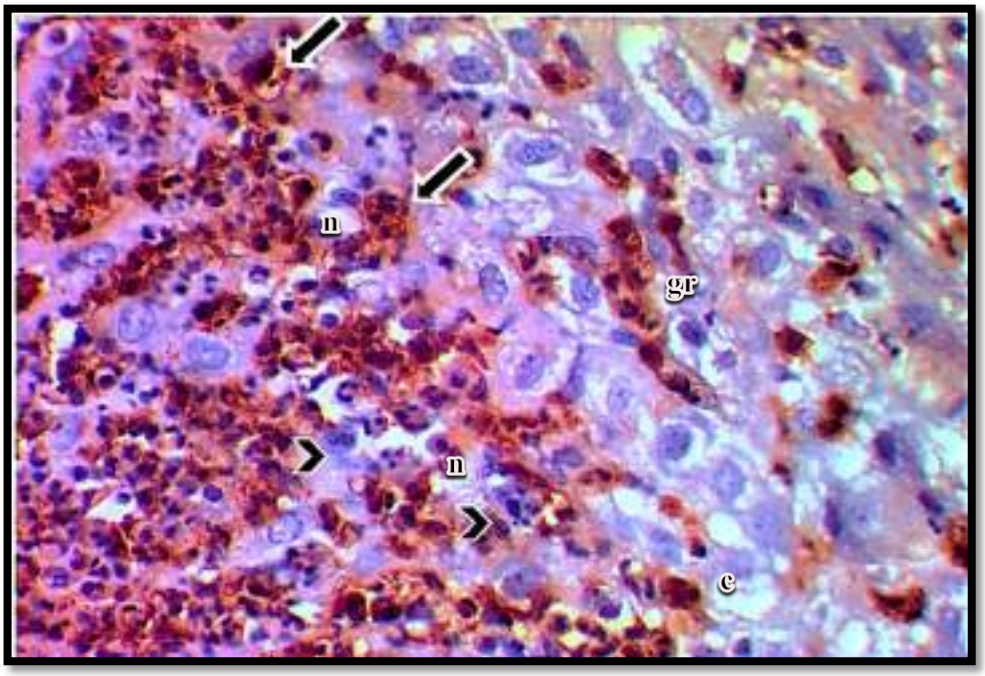

Figure 4-7: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) with the presence of inflammatory cells (arrowhead). Hematoxylin and DAB by IHC. (40X10)X. c=pseudocyst, in=inflammatory cells, n=necrosis, gr=aggregation of *T. gondii*

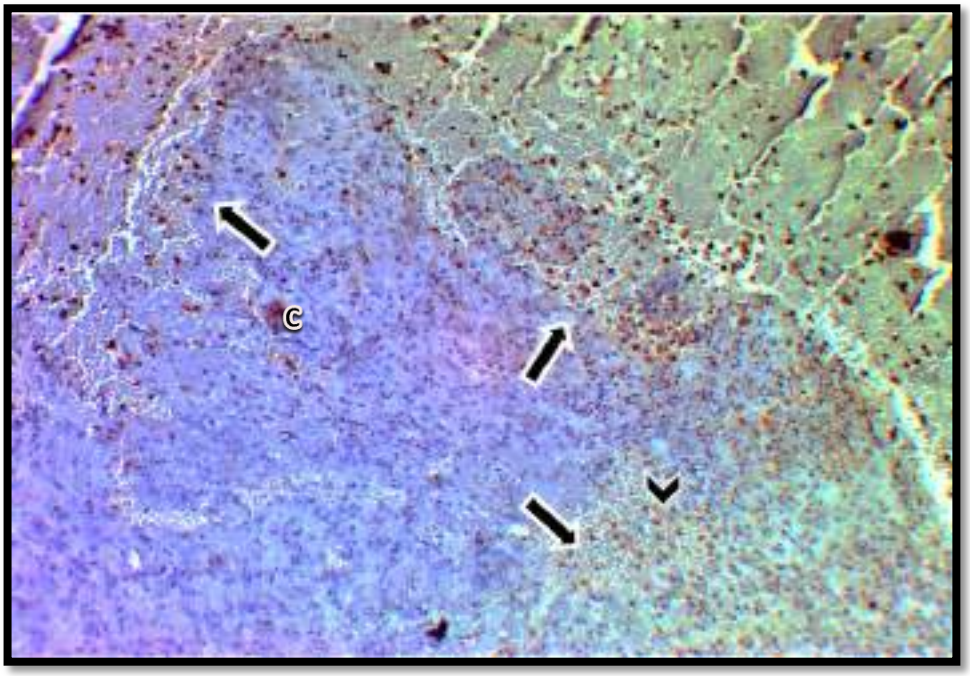

Figure 4-8: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed within epithelial cells of the placenta and hemorrhagic (arrowhead) affected areas. Hematoxylin and DAB by IHC. (10X10)X. C= pseudocysts.

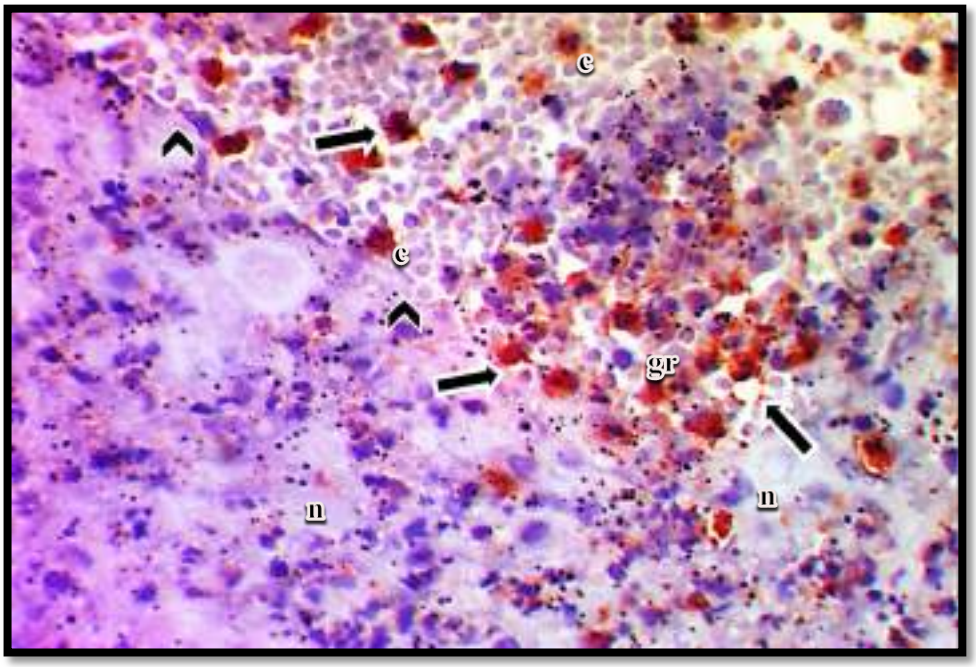

Figure 4-9: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed RBC in hemorrhagic (arrowhead). Hematoxylin and DAB by IHC. (40X10)X. n=necrosis, d=degration, c=pseudocysts, gr=aggregaration of pseudocyst of *T. gondii.*

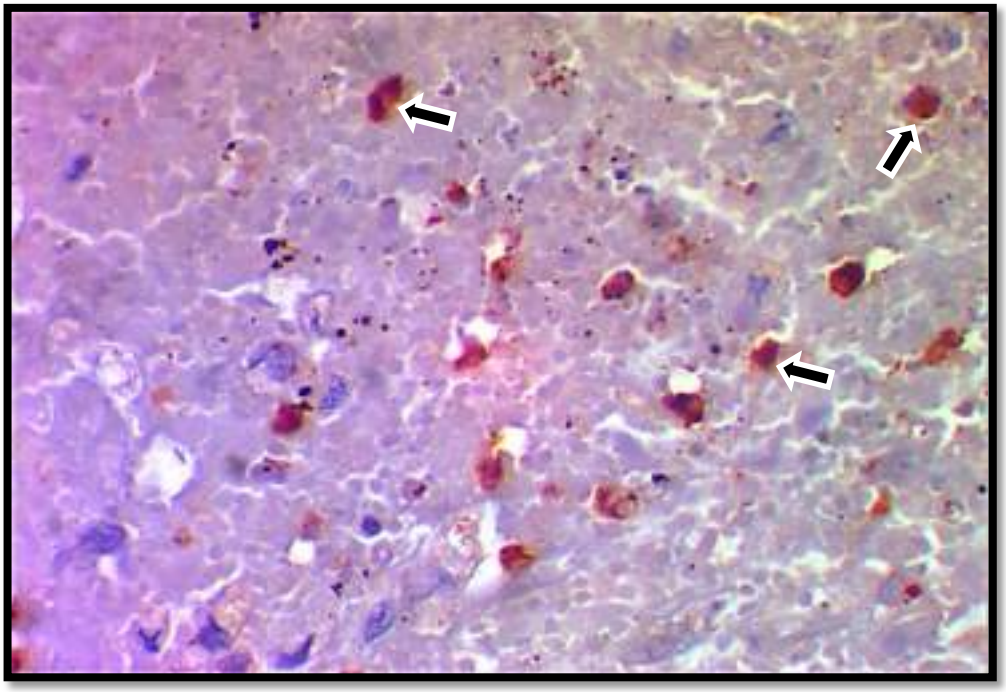

Figure 4-10: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed pseudocyst within epithelial cells of the placenta. Hematoxylin and DAB by IHC. (40X10)X.

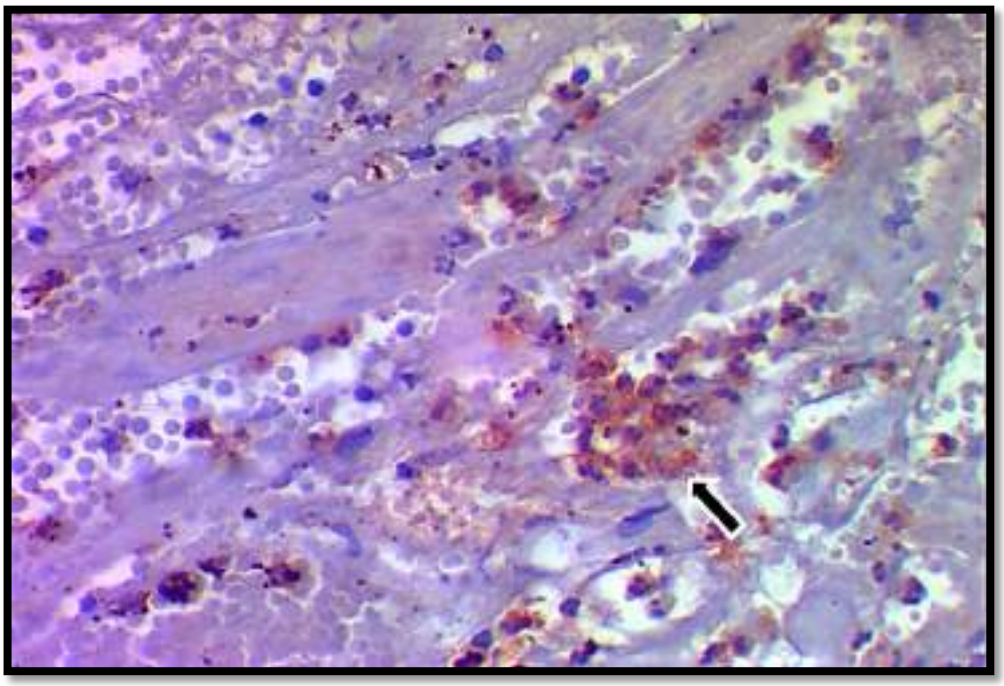

Figure 4-11: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed as aggregations in sinusoids of the placenta. Hematoxylin and DAB by IHC. (40X10)X.

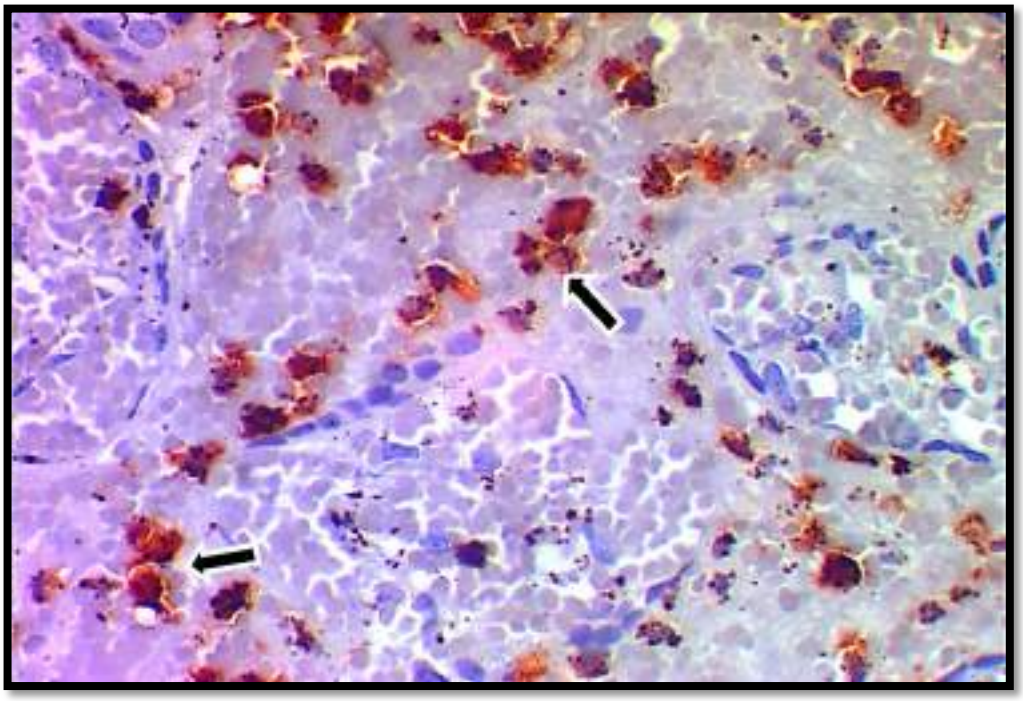

Figure 4-12: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed parasite within a pseudocyst in placenta sinusoids. Hematoxylin and DAB by IHC. (40X10)X.

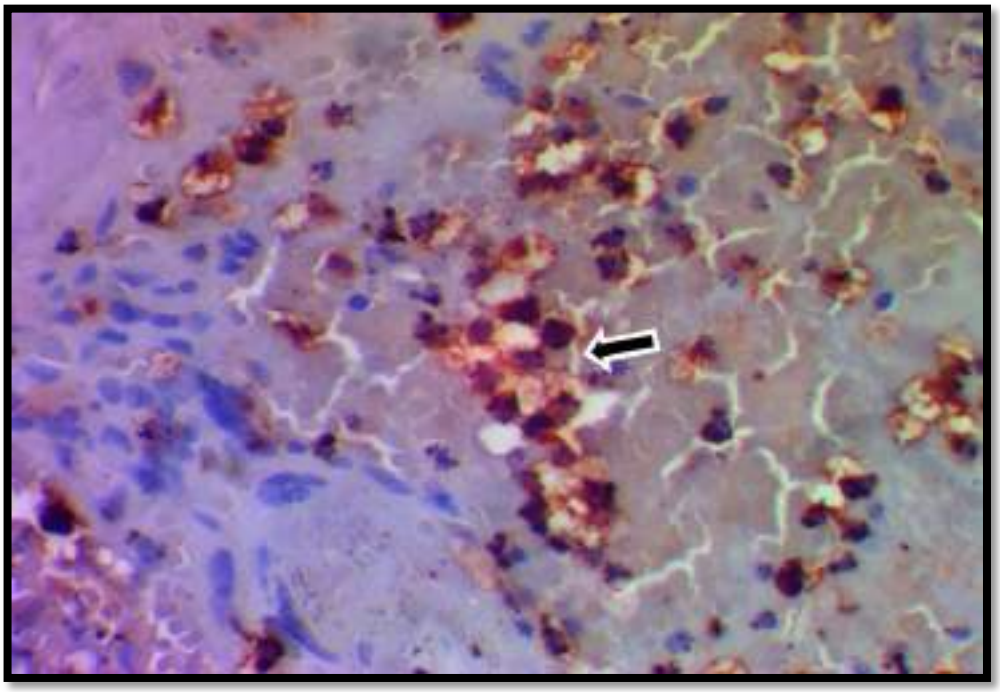

Figure 4-13: Positive expression of anti-*Toxoplasma gondii* primary antibody (arrow) observed pseudocyst as aggregation within placenta sinusoids. Hematoxylin and DAB by IHC. (40X10)X.

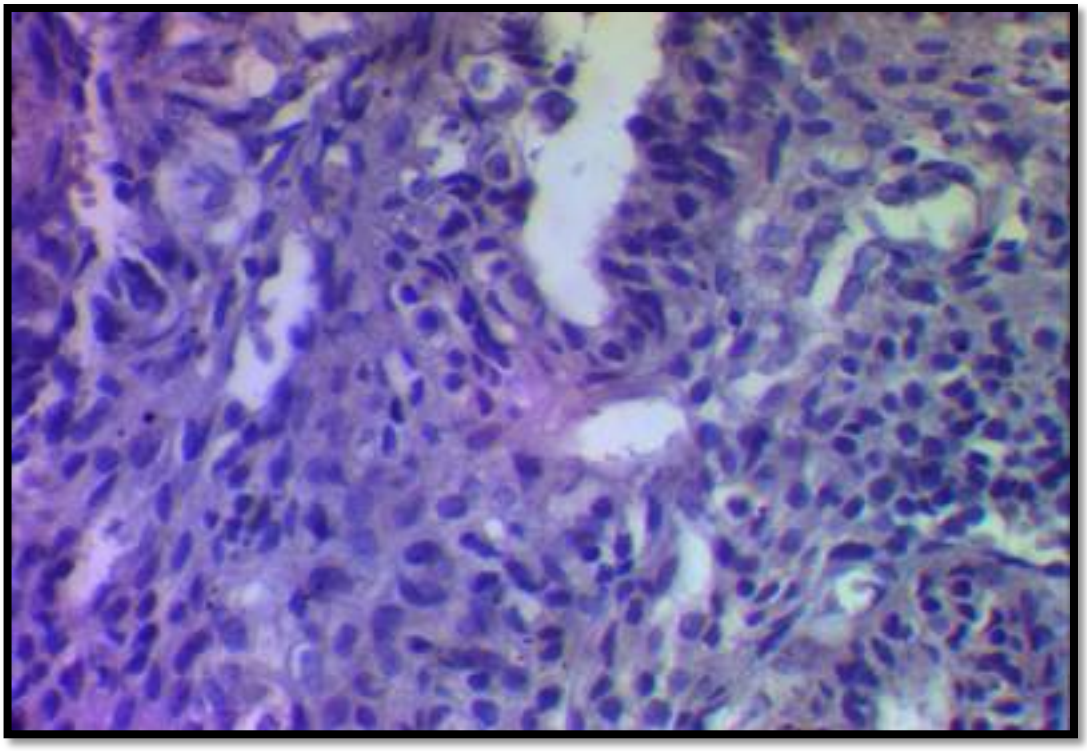

Figure 4-14: No expression of anti-*Toxoplasma gondii* primary antibody in the placenta. Hematoxylin and DAB by IHC. (40X10)X.

4:4 Histopathology test:

The histopathology study of 25 placenta of participants women is shown in (Table 4-6) that all aborted women (100%) were infected with *T. gondii*.

While, it does not see any aborted women among uninfected, (0.0%)

Table 4-6: The percentage of histopathology test of aborted and non-aborted women and toxoplasmosis infection.

Toxoplasmosis		History of Abortion		Total	χ^2	*p*
		Yes (%)	No (%)			
Infected	No.	7(100)	0(0.0)	7(28)	25.00	<0.001
	(%)	100.0	0.0	100		
Uninfected	No.	0(0.0)	18	18 (72)		
	(%)	0.0	100	100		
Total	Count	7	18	25		
	%	28.0	72.0	100.		

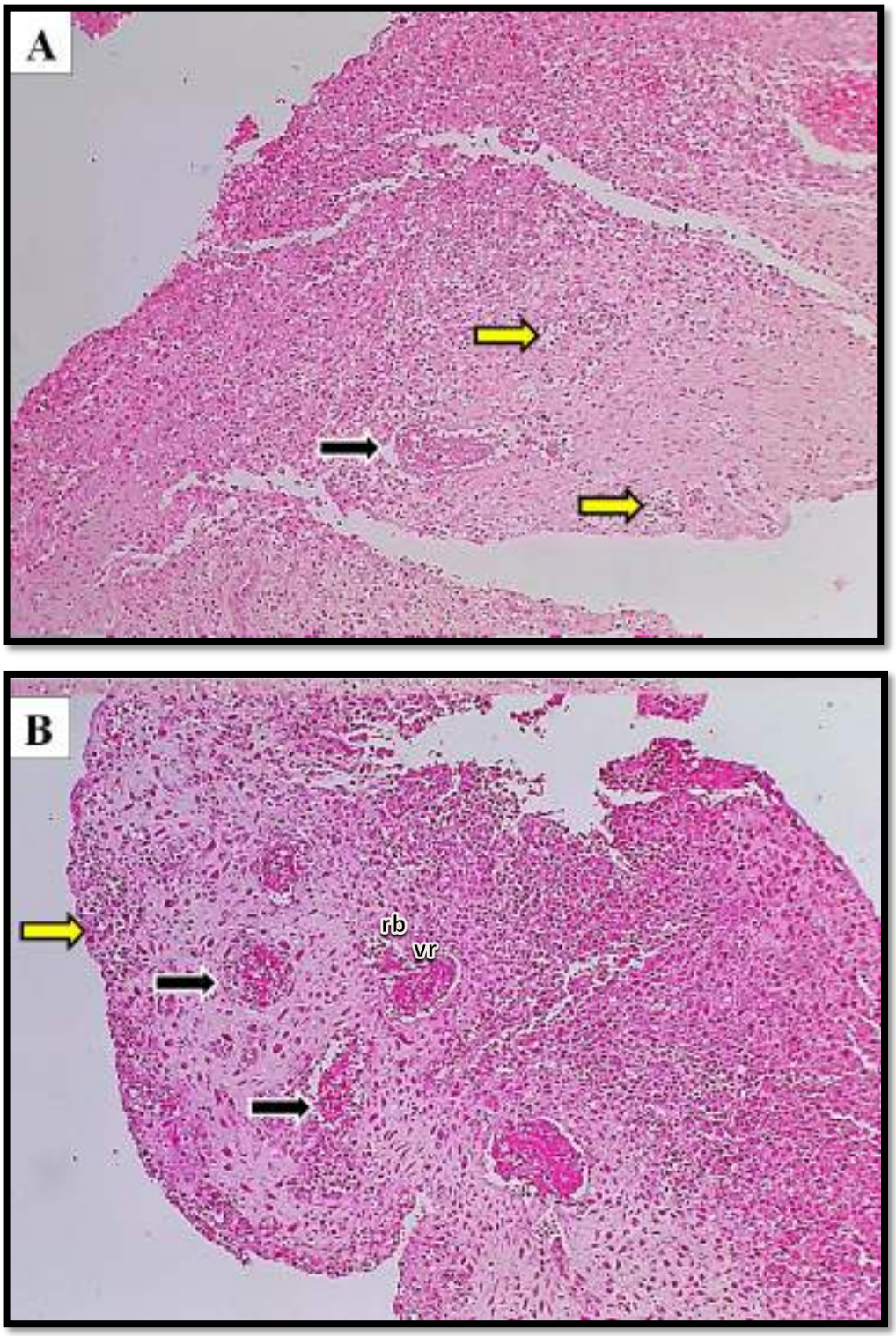

Figure 4-15: Necrosis of decidua cells in placenta tissue. Note necrosis of the decidua cells forming spaces (yellow arrows) in the decidua area especially near blood vessels (black arrows). H&E. A&B: (10X10)X. rb= RBCs and bleeding, vr=blood vessel rupture.

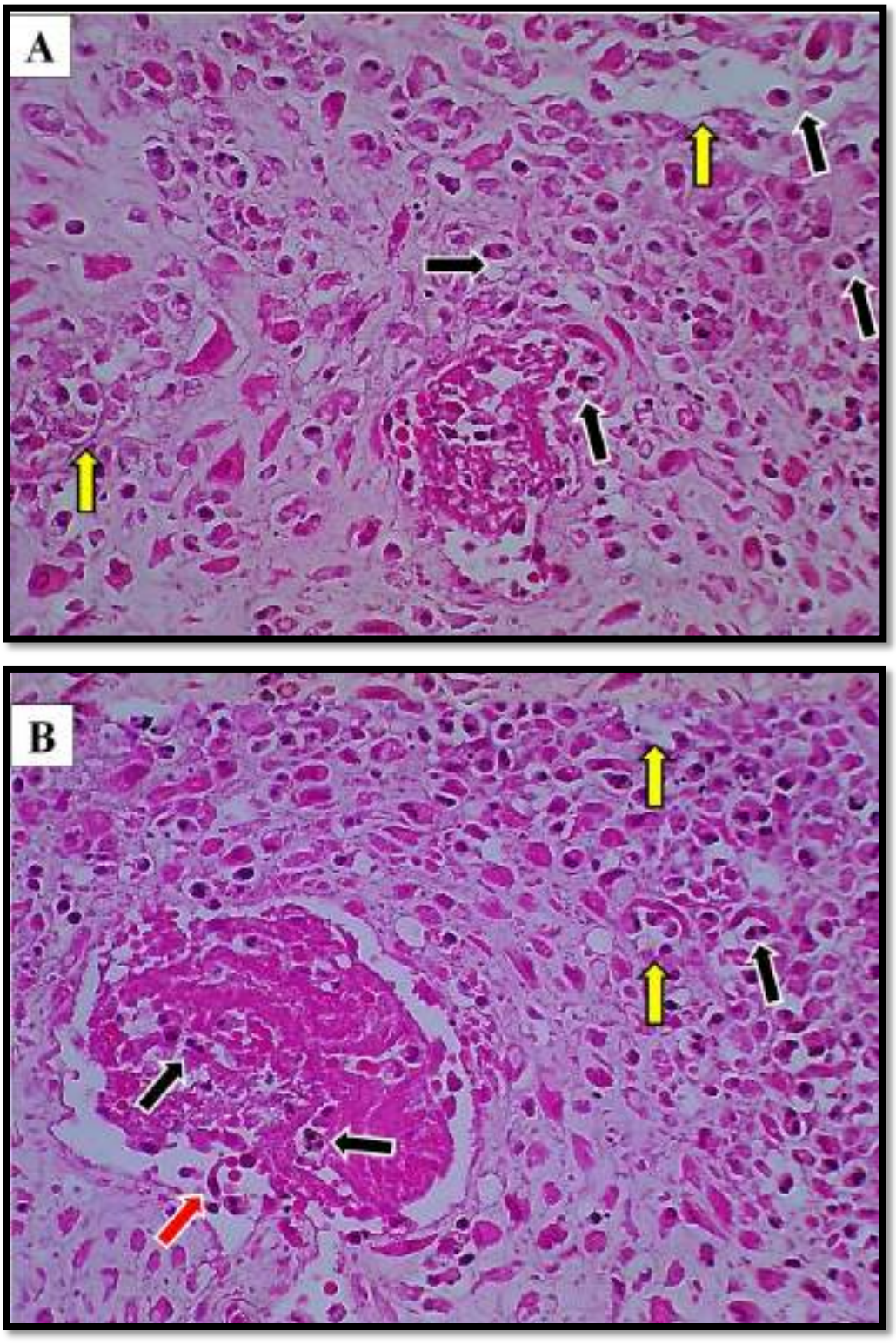

Figure 4-16: Necrosis of decidua cells forming spaces (yellow arrows) around blood vessels in the decidua area. Note tissue cysts (black arrows) and tachyzoite (red arrow) in the blood vessels lumen and within decidua cells. Placenta tissue H&E. A&B: (40X10)X.

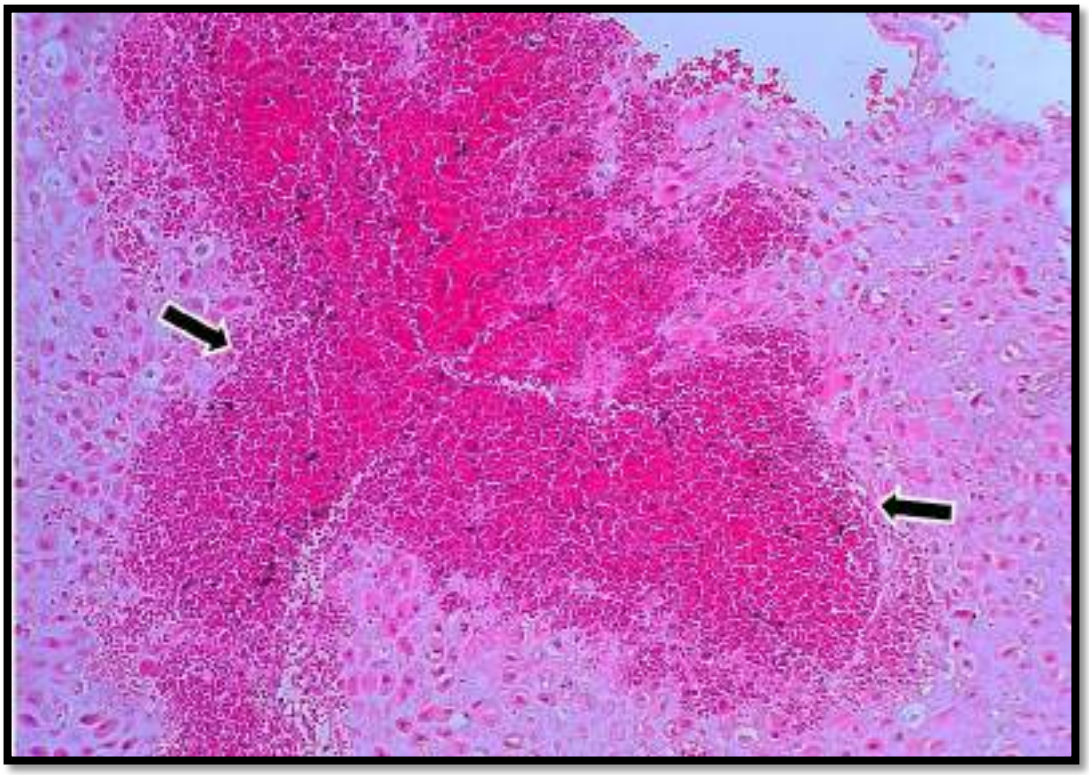

Figure 4-17: Massive hemorrhage (black arrow) in the decidua area that cover the spaces of necrotic cells. H&E. (10X10)X.

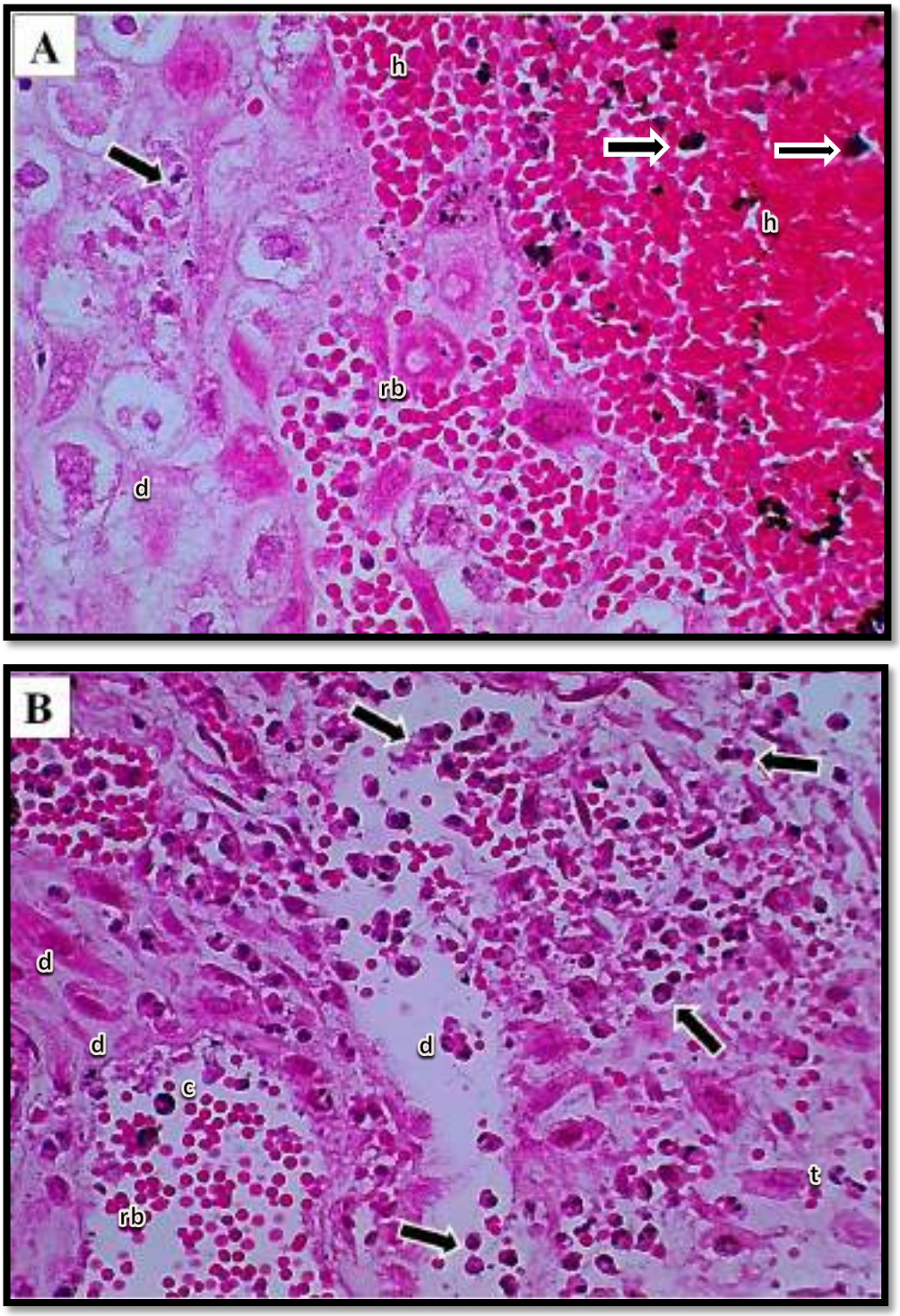

Figure 4-18: Hemorrhage covered the spaces of necrotic cells with the presence of tissue cysts A/(black arrow). tissue cysts B/(black arrow) separated within decidua cells that were found under necrosis. Placenta tissue H&E. A&B: (40X10)X. c=pseudocyst, d=degradation of tissue, rb=RBCs, t=tachyzoite, h=hemorrhage.

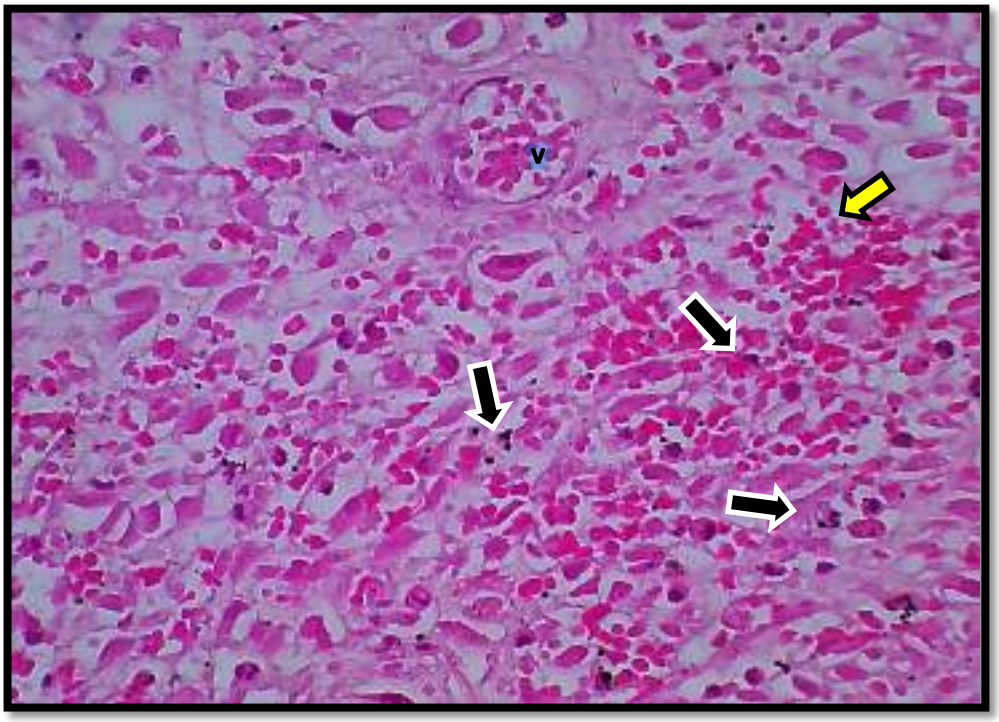

Figure 4-19: Hemorrhage (yellow arrow) that covered spaces of necrotic cells with the presence of tissue cysts (black arrow). Placenta tissue H&E. A: (10X10) and B: (40X10)X. v=blood vessel.

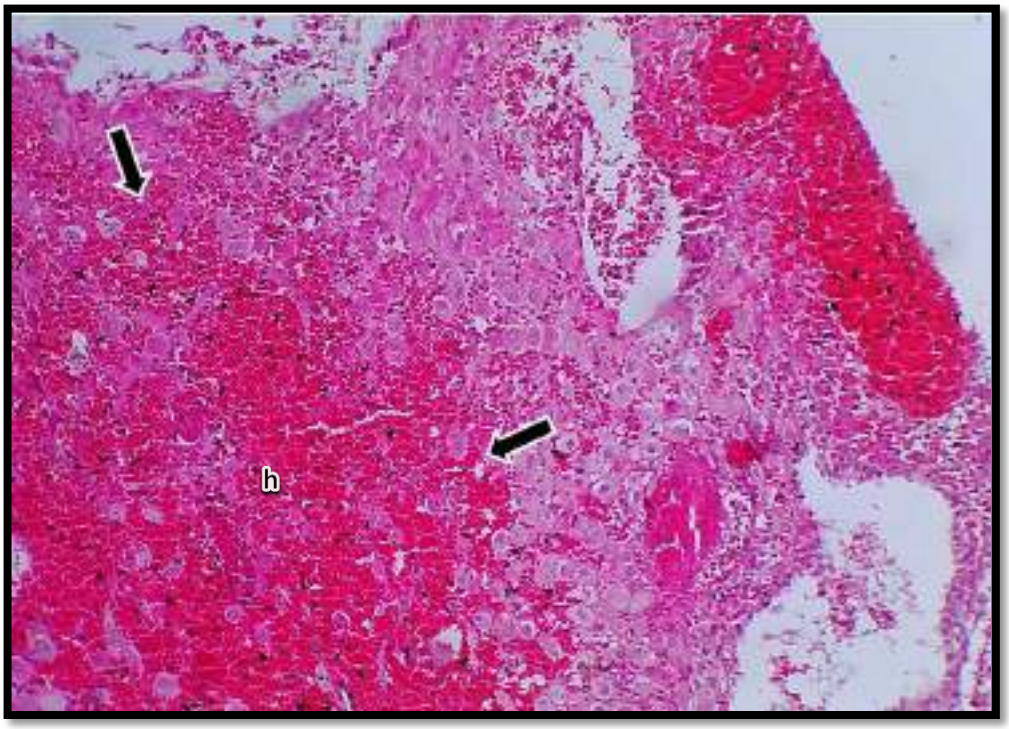

Figure 4-20: Massive hemorrhage (black arrow) in the decidua area that coves the spaces of necrotic cells. Placenta tissue H&E. (10X10)X. h=hemorrhage.

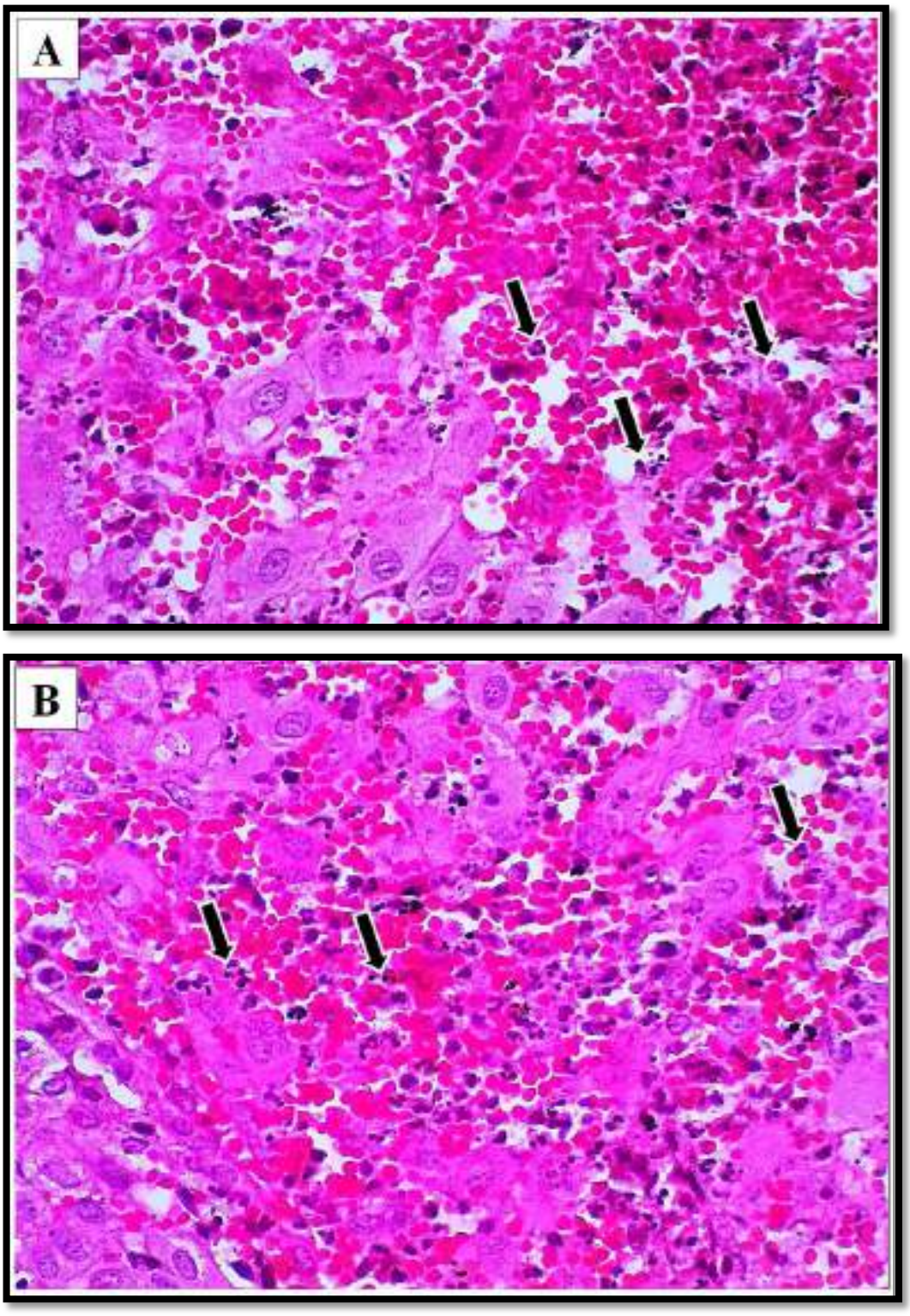

Figure 4-21: Hemorrhage covered the spaces of necrotic cells with the presence of pseudocyst (black arrow). Note the individual decidua cells within the hemorrhage. H&E. A&B: (40X10)X.

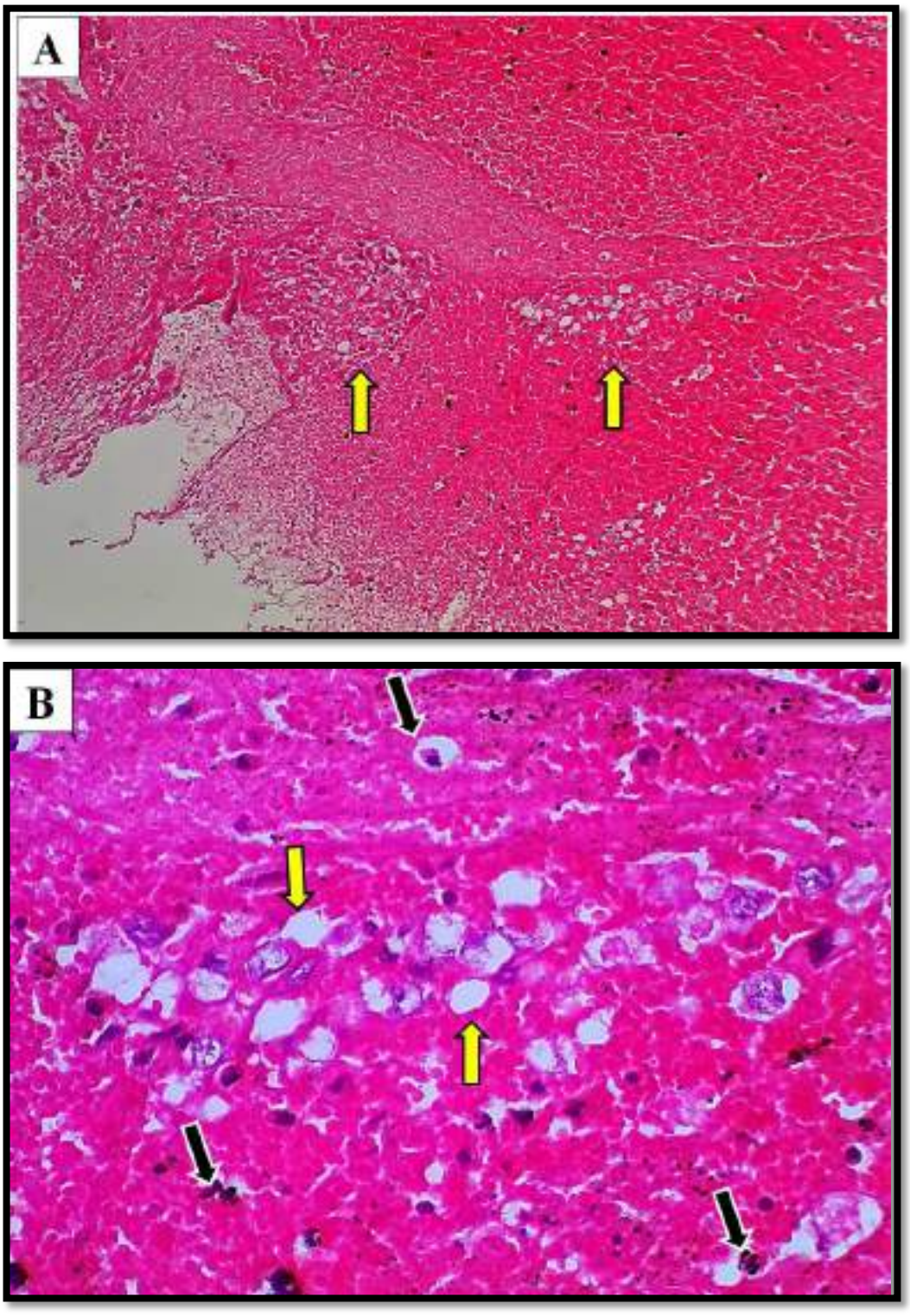

Figure 4-22: Necrosis of basal plate cells forming spaces (yellow arrow) with the presence of pseudocyst (black arrow) in the affected area. Placenta tissue H&E. A: (10X10) and B: (40X10)X.

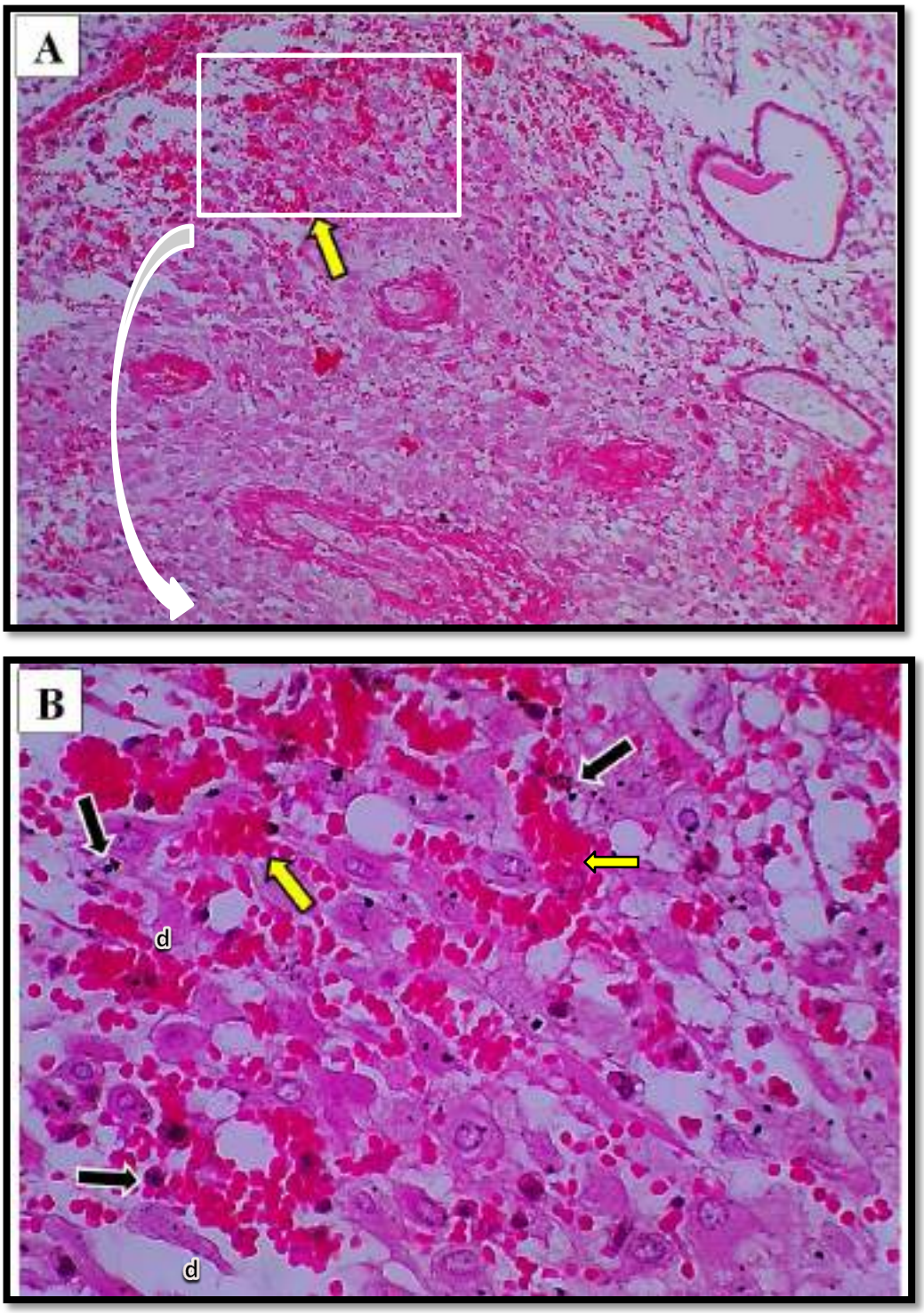

Figure 4-23: Hemorrhage (yellow arrow) covered the spaces of necrotic cells with the presence of pseudocyst (black arrow). Placenta tissue H&E. A: (10X10) and B: (40X10)X. n=necrosis, d=degradation.

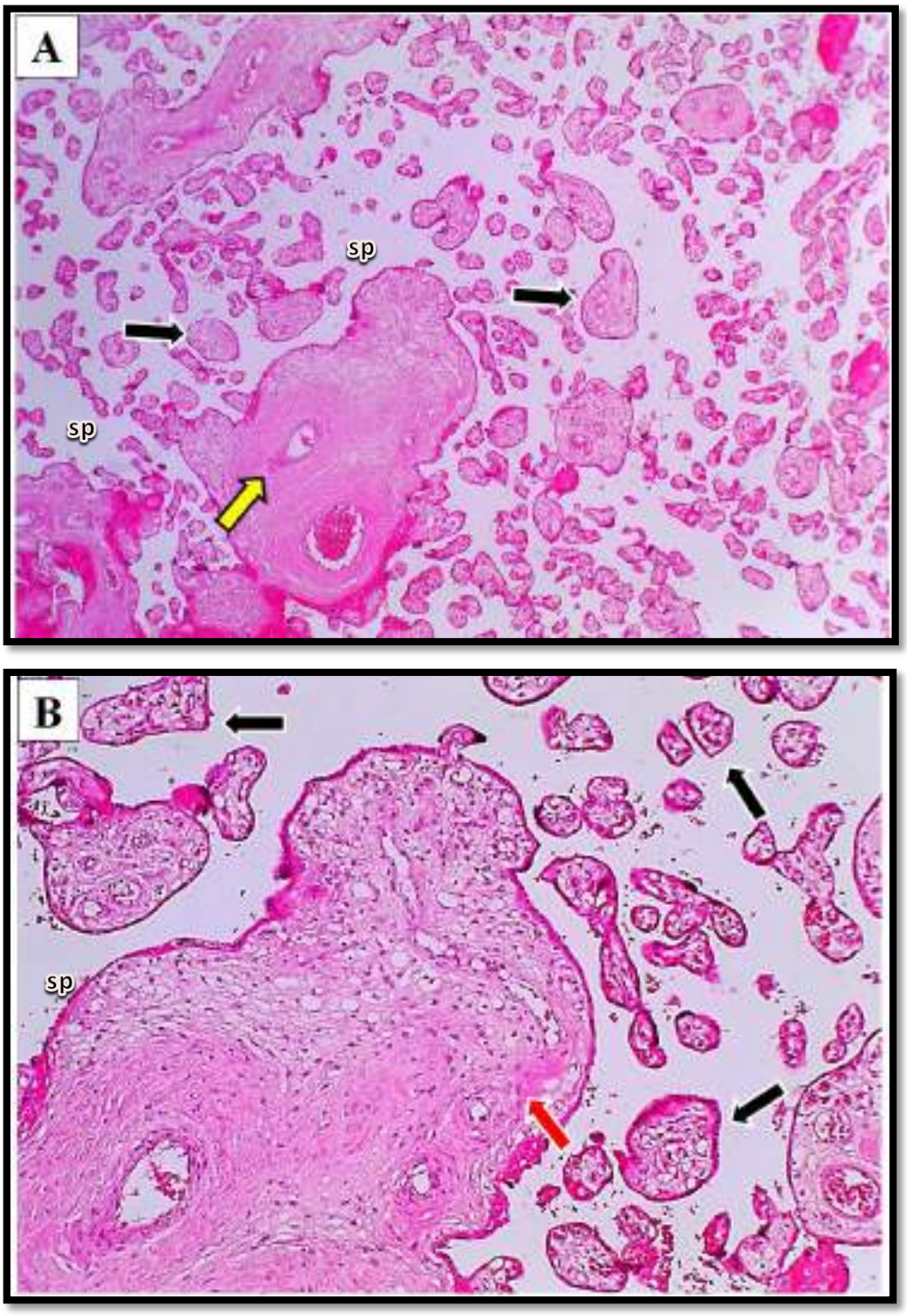

Figure 4-24: Normal placenta architecture. Note villi (black arrow), placental arteries (yellow arrow) and decidua area (red arrow). H&E. A: (4X10) and B: (10X10)X. sp=intervillous space.

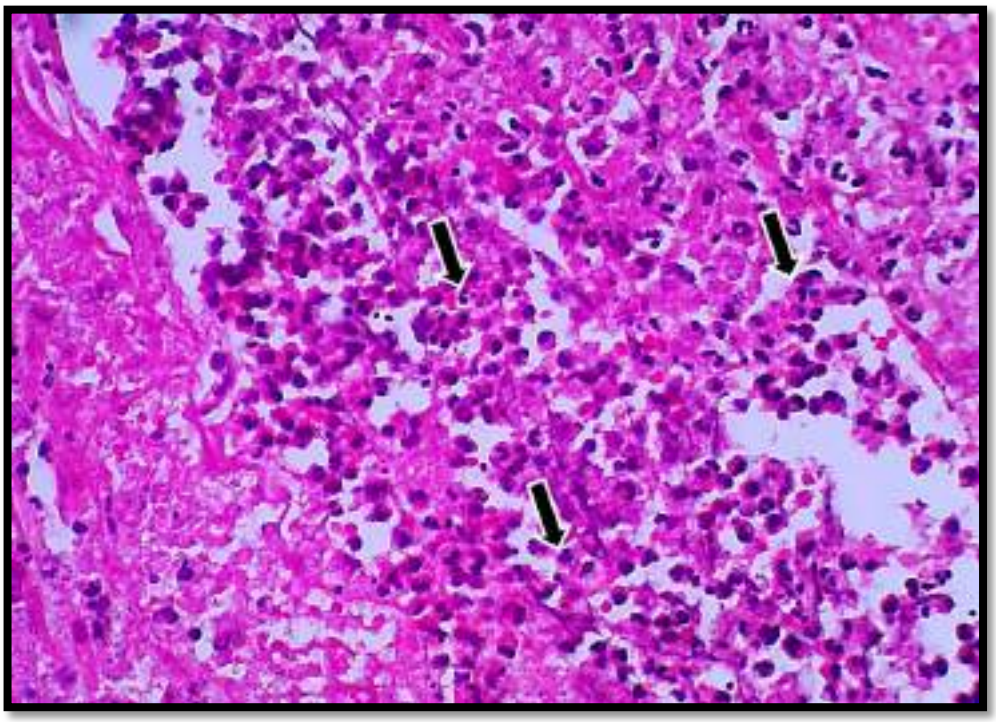

Figure 4-25: Presence of tachyzoite (black arrow) within decidua cells led to necrosis of these cells, where the aggregation of tissue cysts occupied the spaces that formed by necrosis decidua cells. Placenta tissue H&E. B: (40X10)X. d=degradation, n=necrosis.

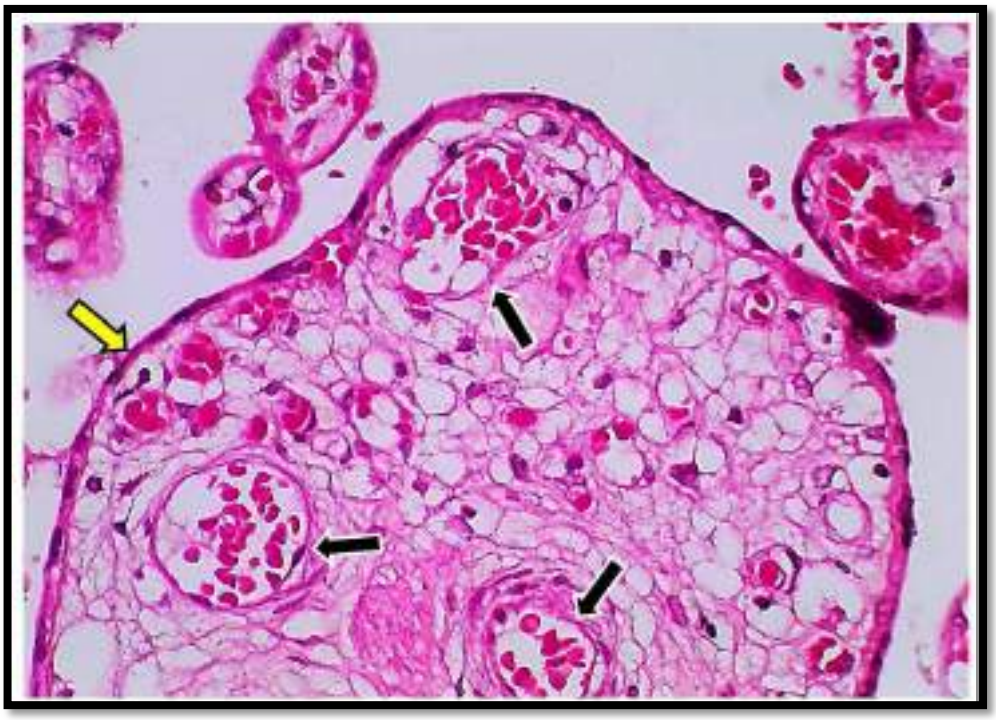

Figure 4-26: Normal architecture of placenta villi. Note capillaries (black arrow) and simple cuboidal epithelium (yellow arrow) of villi. H&E. (40X10)X.

It was observed that the *T. gondii* parasite causes histological damage to the infected tissues of the placenta, including cellular necrosis, in the region of the decidua, and the occurrence of bleeding through the affected areas of blood vessels, and it is seen in Figures 4-19, 4-20 and 4-22, the presence of severe bleeding in the affected placenta and near the blood vessels as well as in the necrotic areas with the presence of the parasite freely outside cells or inside macrophage cells. Figures 4-16 and 4-20 also show the presence of necrosis areas with the presence of pseudocysts of the parasite in some necrotic areas as well as, the presence of tachyzoite in the lumen of blood vessels (Fig. 4-16). It is noted in Figure 4-25 the presence of large numbers of tychozoite stages of the *T. gondii* inside or outside the cells with the presence of pseudocysts of this parasite in the side part of the placenta with the presence of clear cellular necrosis in the decidua infected area, and this is reinforced by what was seen in Figure 4-23, as the presence of severe bleeding and necrosis in other areas of the placenta with the presence of the parasite, while this damage do not notice in the tissues of the uninfected placenta (Fig. 4-24, 4-26).

These results showed that the necrosis and infiltration of some areas in different tissue of the placenta agreed with the finding of de Moraes *et al.* (2011) which revealed an inflammatory infiltration and necrosis in the tissues of the placenta, Unzaga *et al.* (2014), de Oliveira *et al.* (2022) showed severe fibrous necrosis with infiltration, and the observation of the current study in line with Xie *et al.* (2022) who noticed severe necrosis in the tissues of the placenta, and aggregation of red blood cells out blood vessels. Zhao *et al.* (2017), Ismael and Salih (2019), and Vargas-Villavicencio *et al.* (2022) found severe

hemorrhage in the infected placenta with *T. gondii*. These destructions of the tissue of the placenta in aborted women indicate that *T. gondii* implicated as a major cause of abortion in addition to choroiditis as well as thrombosis (Castaño *et al.*, 2014). This study found a high density of tachyzoites and tissue cysts in decidua cells and lumen blood vessels see Figures (4-17, 4-19) and this result is in line with the finding of Vargas-Villavicencio *et al.* (2016).

T. gondii causes abortion by inciting inflammation, when it invades the placental cells, this leads the immune system in more than one way, some of them are discussed in item 4-3.

This study showed many histological differences in the placenta such as necrosis of decidua cells and free tachyzoite and this agrees with Al-hindawi (2012) and disagrees with Razzak *et al.* (2005) which did not found the parasite in the tissues of the aborted placenta. There is a close relationship between toxoplasmosis and abortion in one case of this study had eight previous spontaneous abortion (Fig.4-20) and this agrees with Sultan and AL-Fatlawi (2016).

It was observed that cells infected with the parasite do not undergo apoptosis in contrast to the cells surrounding the infected cell, they die early, as mentioned previously, and an explanation for this the parasite is not in its interest to kill its host, it may be that abortion is an inflammatory reaction against the parasite, or due to the formation of necrotic lesions that are found in the placenta, which leads decrease the oxygen concentration (hypoxic) and nutrient deficiencies and ultimately to the loss of the fetus (Nash *et al*., 1998; Arranz-Solís *et al*., 2021).

Pregnancy and the preservation of the fetus constitute one of the biggest challenges facing the relationship between the mother and her fetus. The fetus constitutes an immunological challenge for the pregnant mother because it is considered in the sight of the immune system as a foreign body (non-self), and therefore all the concepts that control the course and reactions of the immune system tend to eliminate this foreign body, as the body confronts other foreign bodies such as organ transplants or malignant tumors, and the theoretical possible outcomes, the body works to pronunciation or analysis and absorption the embryo at the first moments of its formation, in his creation in preserving embryos for maintaining security and an acceptable balance in contact regions between the mother and her fetus while preserving all the immune capabilities available to the mother to defend herself and her fetus against potential attacks of pathogens that invade that area (Ander *et al.*, 2019).

Chapter Five

Conclusions

&

Recommendations

5:1 Conclusions:

From the findings of the current study, it can be concluded the following:

1. Toxoplasmosis is related to abortions in pregnant women in Maysan province, Iraq.
2. Immunohistochemical technique is a highly sensitive for diagnosis of *T. gondii* than serological (LFIA) and molecular methods and the LFIA may be useful in the initial diagnosis of toxoplasmosis.
3. All three global genotypes I, II, and III of *T. gondii* are recorded in aborted women, and genotype I is the most common than other two genotypes.
4. The IHC technique and histopathological test revealed that the toxoplasmosis caused much damge to the placenta tissue including necrosis, destruction, fibrosis, and blood vessel distraction and they clearly marked the *T. gondii* stages inside the damage tissue.
5. The damage of the aborted placenta are due to the multiplication of the parasite in soft tissues, and the bleeding was due to the destruction of the blood vessel walls by *T. gondii*.
6. The RT-qPCR technique could not be able to detect *T. gondii* DNA in the blood of aborted women.
7. Toxoplasmosis may be associated with recurrent abortions because most women infected with this parasite previously suffered many abortions.

5:2 Recommendations:

1. Carrying out across section study to investigate the overall prevalence of *T. gondii* in Maysan communities and its main transmission mode, because the previous studies are few and limited to one or two regions rather than anther.
2. Spread health awareness among community members, especially women of Iraqi society in general and the Maysan community in particular about the dangers of the parasite, through seminars and awareness programs through print, audio and visual media, as well as through social media.
3. Annual examination of farm and domestic animals such as sheep and cows (intermediate hosts) because they transmit the infection to humans through their meat or milk and cats and dogs.
4. Developing a program for pregnant women's care and conducting the serological test for early treatment of toxoplasmosis.
5. Monitoring the pregnant women through measuring the levels of some immune cytokines like IFN-γ , TNF-α, and IL-10 to protect the fetus from *T. gondii* infection.
6. Ensure that the placentas of aborted women are burnt, so that the cats do not reach to them reach them and feed on them.
7. Pregnant women should avoid changing the litter box for cats, and it should be changed daily.

Chapter Six

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Appendices

Appendix I: Questionnaire template that use in this study.

Code No.: …… Name:……. Age:……..

Date of collecting samples:…….
Residence:.....

Education level:…… The age of husband :…….

Husband job:……. The No. of current abortion:……

Natural abortion? ……Yes □ no □……..

No. of all abortions:…… Congenital abnormal: birth: Yes □ no □

Blood group:…….. Blood group of husband:……..

No. of healthy children:…… History of toxoplasmosis infection:…

History of toxoplasmosis treatment:……..

Gestational period:…….

No. of married time:……..

Do you have any cat in the house: …Yes □ no □……...

Consent to participate in this study:……

The toxoplasmosis result diagnosis:……...

LIFA:…….. Infected □ Non-infected □

RT-qPCR:……... Infected □ Non-infected □

IHC:…… Infected □ Non-infected □

Histopathology:…….. Infected □ Non-infected □

الملخص:

أجريت هذه الدراسة للتحقق من العلاقة بين داء المقوسات Tox*oplasma gondii* والإجهاض لدى النساء اللواتي تم إدخالهن إلى مستشفى الصدر التعليمي بمحافظة ميسان ، العراق ، للفترة من تشرين الثاني (نوفمبر) 2021 إلى أيار (مايو) 2022.

أُجريت هذه الدراسة باستخدام خمس طرق مختلفة للتحقق من قدرتها بما في ذلك المقايسة المناعية للتدفق الجانبي المصلي، والكِيمْياءُ النسيجية المَناعِيَّة، التشريح المرضي النسيجي، وتفاعل البوليمريز المتسلسلة الكمي في الوقت الحقيقي ، وتعدد أشكال طول جزء التقييد للتعرف على الأنماط الجينية لطفيلي المقوسة الكوندية.

وجدت أن هذه الطرق في قدرتها على اكتشاف الطفيلي المقايسة المناعية للتدفق الجانبي المصلي 55٪ ، الكِيمْياءُ النسيجية المَناعِيَّة 80٪ ، التشريح المرضي النسيجي28٪ و تفاعل البوليمريز المتسلسلة الكمي في الوقت الحقيقي 10٪ لأنسجة المشيمة و 0٪ لعينات الدم ، ووجدت أن النمط الجيني "I" هو الأكثر شيوعًا من الطرز الوراثية الثانية والثالثة (50٪ ، 33.33٪ ، 16.67٪) على التوالي. وأظهر الكِيمْياءُ النسيجية المَناعِيَّة كان الاختبار الأكثر حساسية وموثوقية.

اظهرت الشرائح النسيجية التي تم فحصها من قبل الكِيمْياءُ النسيجية المَناعِيَّة و التشريح المرضي أن الإصابة المقوسة الكوندية تسببت في أضرار جسيمة بما في ذلك النخر وتدمير الأنسجة والأوعية الدموية المصابة والنزيف والورم الحبيبي والتليف في الساقط والسدى والأرومة الغاذية ، والزغابات المشيمة ، ذات الكثافة العالية لمراحل المقوسة الكوندية في موقع العدوى.

خلصت الدراسة إلى أن هناك علاقة معنوية بين الإصابة بطفيلي المقوسة الكوندية والإجهاض عند النساء.

وزارة التعليم العالي والبحث العلمي

جامعة ميسان

كلية العلوم

قسم علوم الحياة

الكشف الجزيئي والتغيرات الهيستولوجية المناعية للمشيمة التي تسببها المقوسة الكوندية في النساء المجهضات في محافظة ميسان، العراق.

دراسة مقدمة الى

مجلس كلية العلوم / جامعة ميسان

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل الطالبة

صفاء سلمان داود اللامي

بكالوريوس علوم الحياة/ كلية العلوم

جامعة ميسان 2019

بأشراف

أ.د حسين علي مهوس الساعدي

1444هـ **2023م**