Deanship of Graduate Studies

Al-Quds University

"Identification of immunoreactive *Toxoplasma gondii* epitopes selected by high antibody titer positive human sera suitable for immunodiagnostic techniques"

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Jerusalem – Palestine

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Dedication

I dedicate this work to my Parents

I dedicate this work to my dear husband Ashoor,

I dedicate this work to my Lovely Kids; Monya, Jana and Mazen,

I dedicate this work to my sisters and my brothers,

I dedicate my work to all teachers who teach me,

I dedicate my work to my friends.

Declaration

I certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other University or institution.

Signature:

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Date: 22/12/2016

Acknowledgment

Abstract:

Toxoplasma gondii is a coccidian obligatory intracellular protozoan parasite that utilizes cats as a definitive host and it is the causative agent of the toxoplasmosis (Abu-Madi, Behnke et al. 2008). The parasite affect about 30% of human population and it is considered a major zoonotic disease. (Chunlei Sua, Zhou et al. 2012; R. Edelhofer 2010) The disease is endemic in many countries in the world including Europe, United States and Mediterranean regions. The present study was conducted to identify immunoreactive *Toxoplasma gondii* epitopes using M13 phage display library for identifying peptides that mimic *Toxoplasma gondii* antigens. Phage display was done by plaque assay in 12 amino acid peptide phage library. For phage screening purposes; sera were collected from *Toxoplasma gondii* infected pregnant women, high antibody titer sera were pooled from different patients after screening for anti-*Toxoplasma* crude antigens by Modified Agglutination Test (MAT). By the aid of the high antibody titer *Toxoplasma gondii* pooled sera, three screening cycles of M13 phage display library, (12 amino acids peptides) were achieved. In the third screening cycle; 329 reactive plaques were obtained, 138 plaques that gave the strongest signals with pooled sera were selected for further analysis. Reactive peptides were isolated and sub-cloned in recombinant expression plasmid and were used in dot-ELISA to screen the selected M13 phage clones with pooled sera. ELISA test was also carried out to detect *Toxoplasma* infected individuals were identified. Phage Dot-Blot Assay standardized for the confirmation of reactivity selected M13 phage clones with *Toxoplasma gondii* high antibody titer pooled sera and ELISA Assay revealed reactivity between selected amplified phages and some collected sera of *Toxoplasma gondii* infected individuals. The DNA coding the 12 amino acid peptide fused with the PIII M13 phage gene was amplified using specific primers flanking the peptide designed based on the PIII gene. DNA sequence was determined for 24 different clones, followed by bioinformatics

DNA analysis by means of DNA alignment and BLAST search analysis. From the 24 sequenced peptides, 19 selected peptide clones represent the selected M13 phages showed no direct similarity to *Toxoplasma gondii* parasite DNA genomic sequences. So, due to that we was searching on amino acids sequence for the selected M13 phages through BLAST program. Data analysis showed a cross-reactivity between the selected M13 phages and anti-*Toxoplasma* pooled sera; which the sequences represent the selected M13 phages related to protozoan parasites such as *Plasmodium knowlesi* and *Plasmodium fragile*. These protozoan parasites are closely related to *Toxoplasma* family (Sarcocystidae). The selected M13 phage clones were pooled and tested for their reactivity against pooled anti-*Toxoplasma* sera in dot-blot and ELISA format. The obtained results showed specific interaction that need further studies and evaluation for the use of these phages in future *Toxoplasma* diagnosis.

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Abbreviations Used:

AIDS	Acquired Immune Deficiency Syndrome
CIR	Cellular Immune Response
CMI	Cell Mediated Immunity
D.H20	Distilled water
D.D.H20	Double Distilled water
DAB	Diaminobenzidine
E. coli	*Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
FCS	Fetal Calf Serum
GRAs	Dense granule proteins
HRP	Horseradish peroxidise
IPTG	Isopropyl-beta-D-thiogalactoside
IL	Interleukin
kDa	Kilo Dalton
MOI	Multiplicity of infection
2-ME	Mercapoethanol
MAT	Modified Agglutination Test
NK	Natural Killer
OPD	O- phenyldiamine
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline-Tween 20
PV	Parasitophorous vacuole
PCR	Polymerase Chain Reaction
RF	Rheumatoide Factor
ROP	Rhoptries proteins
RPDL	Random phage display libraries
RT	Room temperature
SDS-PAGE	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis
TBST	Tris-buffered saline and Tween 20
TM-MICs	Transmembrane micronemal proteins
TNF	Tumor necrosis factor

Introduction:

Toxoplasmosis, is a disease that affects human and animals generally, but it has a stronger effect and more dangerous to women in child bearing age. Toxoplasmosis caused by parasite named *Toxoplasma gondii* that has a complex life cycle in the reproduction of stray cats that feed on rodents and birds, therefore that disease called toxoplasmosis.

Toxoplasma gondii is a coccidian obligatory intracellular protozoan parasite that utilizes cats as a definitive host and it is the causative agent of the toxoplasmosis disease (Abu-Madi, Behnke et al. 2008) . The parasite affect about 30% of human population and it is considered a major zoonotic disease of human that also infects all warm-blooded vertebrates including mammalian and birds (Chunlei Sua, Zhou et al. 2012; R. Edelhofer 2010).

Furthermore, the disease is endemic in many countries in the world including Europe, United States and Mediterranean regions. The geographic distribution of toxoplasmosis varies widely between different parts of the world and attributed to climate, anthropogenic factors and the available water quality. Cold climate parts of the hemisphere have a low sero-prevalence (10-30%) compared to humid and warm climate tropical countries. (Mohammed 2011; Gangneux F and Drade 2012) Toxoplasmosis distribution is also affected by the genotype of the infecting strain, Type I *Toxoplasma* is highly virulent, while types II and III strains are less virulent. (Dalimi and Abdole 2012)

In addition, toxoplasmosis is considered as a public health concern mainly in developing countries. The disease has been reported in almost all the world that affect up to one third of the human population in the world including the Mediterranean regions. Previous studies have identified the prevalence of *Toxoplasma gondii* in Palestine cities (Hebron district, and Gaza strip), the studies showed high IgG and IgM antibody titers

among pregnant women. Recent data; based on some Hospital reports in Hebron city, indicates many abortions occur among pregnant women (personal communications).

In this study the development of *Toxoplasma* immunological test based on identification of immunoreactive peptides should become possible. The newly identified peptides should overcome the high antibody titers seen among negative controls examined by different imported ELISA kits. This study will allow the provision of a continuous supply of *Toxoplasma* antigenic material and in sufficient quantities.

PART 1: Literature Review.

1.1. History:

In 1908, the *Toxoplasma gondii* was first discovered by Nicolle and Manceaux in the tissues of a hamster-like rodent. (Gebremedhin 2014) At the same time in Brazil, Splendore (1980) described a similar parasite in a rabbit that had died with paralysis. Splendore erroneously identifying it as *Leishmania*. (Gebremedhin 2014; Sousa 2009)

The first case of toxoplasmosis in human was reported in 1923 by Janku who described the cysts in the retina of a child with congenital infection (microphtalmia and hydrocephalus). (Hay, Hutchison et al. 1981; Sousa 2009)

A fatal case of infantile granulomatous encephalitis that was identified in 1937 in Wolf and Cowen by Sabin and Olitski. Depending on their studies *Toxoplasma gondii* was a cause of congenital disease in Wolf and Cowen. (Sousa 2009)

Until 1939, the medical importance of *Toxoplasma gondii* remained unknown when it was identified in the tissues of infected infant had classic traid of symptoms (hydrocephalus, retinochoroiditis and intracranial calcification). (Gebremedhin 2014) The first time an acquired case of toxoplasmosis in a young man was described by Weinman and Pinkerton in 1940. (Sousa 2009) In 1948, Sabin and Feldman discovered the *Toxoplasma gondii* antibody test (dye test); which is the most serological test for toxoplasmosis to detect human infection. This discovery led to the recognition that the *Toxoplasma gondii* is a common parasite of warm blooded host. (Gebremedhin 2014; Sousa 2009)

The importance of veterinary became known in 1957 when *Toxoplasma gondii* was found to cause abortion in sheep. Coccidian nature of *Toxoplasma gondii* came first from electronic microscopic examination carried out in 1960. (Gebremedhin 2014) Hutchison revealed that the cat was the definitive host when he found oocysts in feline feces in 1969. (Sousa 2009) In 1970, the life cycle of *Toxoplasma gondii* was defined by the discovery of the sexual phase in the small intestine of cat. *Toxoplasma gondii* described as a major

cause of death in AIDS patients in 1980, 1981 and 1982; clarifying the importance of the immune system in controlling *Toxoplasma gondii* infection. (Gebremedhin 2014; Sousa 2009)

Nowadays the parasite is found widely distributed in the world and in warm blooded animals including humans. (Gebremedhin 2014)

1.2. Taxonomy:

The phylum Apicomplexa includes pathogens that have a medical and veterinary importance such as *Toxoplasma*, *Plasmodium*, *Neosopra* and *Eimeria*. (Gebremedhin 2014)

Table 1.2.1: Taxonomy of *Toxoplasma gondii* parasite

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Apicomplexa
Class	Sporozoasida
Order	Eucoccidiorida
Family	Sarcocystidae
Genus	*Toxoplasma*
Species	*Gondii*

1.3. Biology:

1.3.1. Life cycle and transmission:

Toxoplasma gondii life cycle includes intestinal and extra-intestinal stages; the intestinal stage occurs in cats that is the only definitive host and excretes oocysts (Fig.1.3.1) the infective stage that passed to the environment with feces material. The extra-intestinal phase occurs in all infected animals including cats and produces the rapid multiplying tachyzoites (Fig. 1.3.2) or bradyzoites tissue pseudocysts. (Gebremedhin 2014; Sousa 2009) *Toxoplasma gondii* oocysts are shed by domestic cats for only a short period (1-2 Weeks) resulting in a wide spread contamination of the environment. (Dubey 2004; Hill and Dubey 2002) Infection by the *Toxoplasma* may be unrelated to direct exposure to a cat (Montoya, 2002), since the parasite can be transmitted horizontally i.e. ingesting tissue cysts or ingesting tachyzoites which are contained in raw or inadequately cooked infected meat, or by ingesting infectious oocysts passed cats' feces. Vertical transmission route results from congenital transmission of the parasite from mother to fetus via the placenta. (Jaiswal, Pokherl et al. 2014) (Fig. 1.3.3)

Figure 1.3.1: *Toxoplasma gondii* sporulated oocyst, Adapted from CDC.
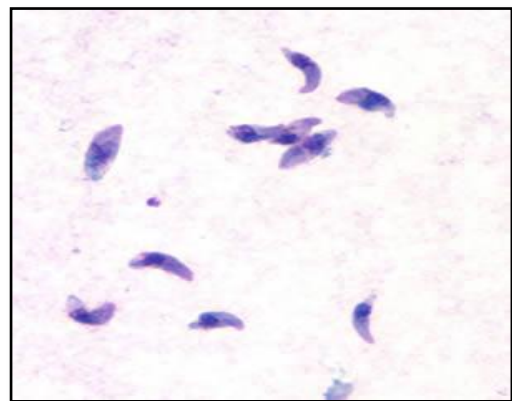
Figure 1.3.2: *Toxoplasma gondii* tachyzoites, Adapted from CDC.
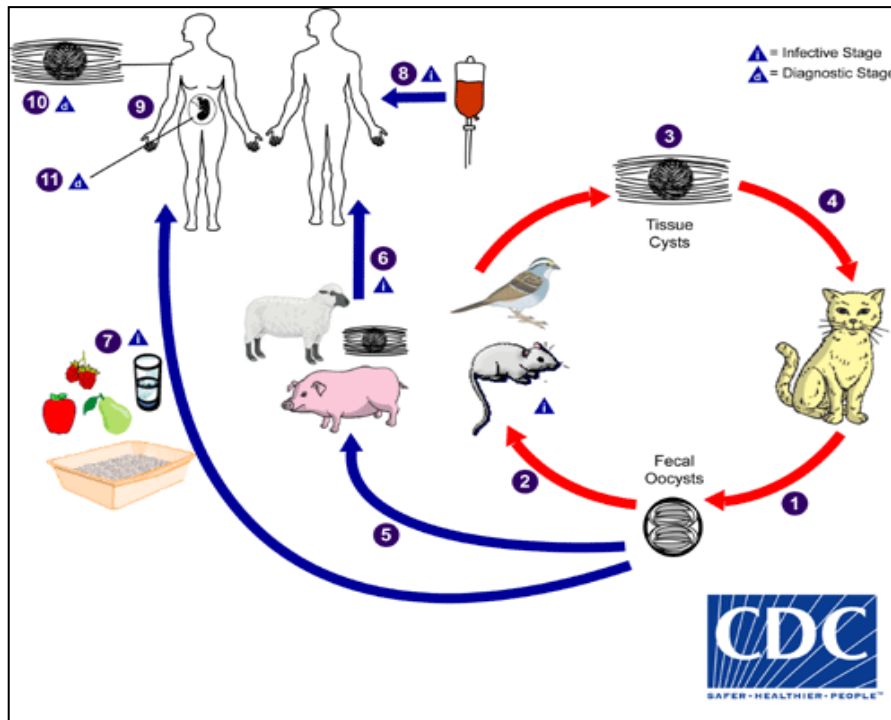
Figure 1.3.3: *Toxoplasma gondii* parasite life cycle, Adapted from CDC.

1.3.2. Population structure and Genotypes:

Highly virulent strains are obtained recently from immunocompetent patients. Genotyping studies on *Toxoplasma gondii* strains describe the population structure of *Toxoplasma gondii* as highly clonal with three main genotypes that differ dramatically in virulence. (Sibly, Mordue et al. 2002) Despite genetically very similar, designated as Type I, Type II and Type III. These types respond to the clonal population structure of *Toxoplasma gondii* and related to mouse-virulence. Since main types were found predominant in other continents that had a complex population structure of *Toxoplasma gondii*; the genotypes not belonging to three main types. (Darde 2008; Sibly, Mordue et al. 2002) Distribution of these types differ from one region to another, that observed during genetic analysis that shown the parasite population structure varies globally. Strains majority were found in North America and Europe. Type I is rarely found in America and Europe, while type II is dominant in North America and Europe, but type III is encountered in Europe. (Gebremedhin 2014) Geographic distribution of human

Toxoplasma gondii genotypes including congenital toxoplasmosis in the world involving Eurpoe, America, Africa and Asia possible relationships with human disease in immunocompetent individuals (table 1.3.2.1).

Table 1.3.2.1: "Geographic distribution of *Toxoplasma gondii* genotypes in the world and possible relationships with human disease". (Gangneux and Marde 2012)

Geographical area	Genotypes	Specific features of human disease in Immunocompetent individuals and those with congenital toxoplasmosis
Europe	- Type II (haplogroup 2) highly permanent. - Type III, more present in South Europe. - Other genotypes sporadically observed.	- Asympotomatic - Benign disease in immunocompetent individuals associated with type II or III. - lower rate of retinochorditis in immunocompetent patients and in those with congenital toxoplasmosis than in areas of South America
North America	- Type II (haplogroup 2). - haplogroup 12. - Type III (haplogroup 3). - Other genotypes	- Asympotomatic . - Benign disease in immunocompetent individuals associated with type II or III. - Not enough data for other haplogroups.
South and Central America	- High genotypic diversity. - Some haplogroups shared with Africa (haplogroup 6). - Type II sporadically present. - Type I rarely encountered. - Highly atypical genotypes in the Amazonian forest.	- Higher rate and severity of retinochorditis in immunocompetent patients and in those with congenital toxoplasmosis disseminated; potentially lethal. - Cases observed with the most atypical genotypes.
Africa	- African 1, 2, 3 (haplogroup 6). - Type III (haplogroup 3). - Type II.	- Data showed a higher rate of retinochorditis than in Europe
Asia	- Less genotypic diversity than in South America. - Type III (haplogroup 3). - A common haplogroup widespread across the continent.	- No comparative data

1.3.3. Pathogenicity and Virulence:

Course of *Toxoplasma gondii* infection is determined by virulence of the *Toxoplasma* organism parasite. Strains of *Toxoplasma gondii* might differ in ability to cross placenta to cause congenital disease due to the genotypes that may differ in virulence and migratory capacity.

Type I is considered the higher virulence due to transmission, migration and penetration vitro biological characteristics. (Gebremedhin 2014) Type III strains are less virulent in mice rather than type I and II (English ED, Adomako et al. 2015); type III is considered as a virulent in mice, but type I leads to widespread parasite and death of mice and mice still survive with type II strains. (Gebremedhin 2014) Expression of virulence needs host response. Many virulence effectors termed core effectors in the *Toxoplasma gondii* enables *Toxoplasma gondii* to evade host defenses. (English ED, Adomako et al. 2015) Type I strains associated with higher virulence in some acquired ocular diseased patient's cases, but in reactivation of chronic infection in immunocompromised patients and with no congenital infection (through crossing placenta) indicates asymptomatic infection immunocompromised patients. (Weiss and Kim 2007)

The most prevalent in human disease, more virulent in immature fetuses and in immunocompromised patients is type II strains. Type II responsible for a symptomatic infection or benign toxoplasmosis. (Gebremedhin 2014; Weiss and Kim 2007) Although there are very few reports of human disease associated with type III genotype; the type III may also be the case for the type II starins. (Weiss and Kim 2007)

1.4. Diagnosis and Identification:

Cysts of *Toxoplasma gondii* may be visualized in tissues by the Wright -Giemsa stain, or by immuno-staining, which is more specific. (Conley, Jenkins et al. 1981; Goldstein,

Montoya et al. 2008) The disease can be diagnosed in human by serological tests that includes the detection of IgM and IgG antibodies, or directly by molecular methods. (Paquet et al. 2013; Goldstein, Montoya et al. 2008; Hill and Dubey 2002) Clinical sings are not sufficient and nonspecific for a definite diagnosis. *Toxoplasma gondii* serological examination is performed using modified agglutination test (MAT). (Al-Adhamia et al. 2016) The MAT test utilizes formalized tachyzoites and it detects only IgG antibodies as mercaptoethanol used in the test destroys IgM antibodies. The test has been reported to demonstrate high sensitivity and specificity when compared to other serological assays. (Shaapan et al. 2008)

Recent infection is confirmed by demonstrating a significant rise in IgM antibody titers in serum sample or by detecting *toxoplasma* DNA in amniotic fluid of the fetus by molecular techniques PCR (Goldstein, Montoya et al. 2008; Nijem and Al-Amleh 2009). Elevation of IgG does not distinguish between infection acquired recently or the distant past (Jones, Wilson et al. 2001), IgM and IgG elevated within 1 to 2 weeks of infection, in acute infection (Montoya, Bennett et al. 2000). The differentiation between acute and a chronic infection based on IgM and IgG antibodies titers is difficult to interpret, and other confirmatory testing is required (Paquet et al. 2013). While, toxoplasmosis screening during pregnancy is based on detection of specific IgG antibodies (Khammari, Saghrouni et al. 2013; Gangneux and Marde 2012). In newborn serum the IgM or IgA *Toxoplasma gondii* antibodies were demonstrated to serologic diagnosis of congenital toxoplasmosis. (Remington, J.S et al. 2004)

Some commercial IgM test kits for toxoplasmosis have had problems with specificity, resulting in high rates of false-positive test results, and these kits have low specificity and the reported results are misinterpreted (Jones, Wilson et al. 2001; Montoya 2002). False positive IgM antibody results may obtained if individual have Rheumatoid

factor (RF) and antinuclear antibodies (Montoya 2002; Abolghasem et al. 2011). Although Enzyme linked Immune-sorbent Assay (ELISA) are mostly common used, the dye test of Sabin and formalin is considered the gold standard for toxoplasmosis diagnosis. However, the dye test is complex to carry out and not suitable for routine use. (Khammari, Saghrouni et al., 2013; Khammari, Saghrouni, 2014)

Polymerase chain reaction (PCR) is a test used to detect the amniotic fluid, PCR has allowed earlier amniotic fluid than fetal blood sampling (Hohlfeld et al. 1994). PCR of amniotic fluid is more sensitive than fetal blood sampling (Foulon et al. 1999; Jones, Wilson et al. 2001).

1.5. Treatment:

The world passes through complications of diseasse, so the better anti- *toxoplasma* need is necessary and urgent (Doggetta et al. 2012). The recommended therapies are based on extrapolations from in vitro studies, animal models mostly murine and on the clinical experience and practice of physicians experienced. (Weiss and Kim 2007) Current anti-*toxoplasma* drugs suppress active infection but does not cure latent infection. (Doggetta et al. 2012) The decision to toxoplasmosis treatment is based on the immune states of the patient and on the weather of the woman with acute toxoplasmosis is pregnant or not. (Weiss and Kim 2007) Toxoplasmosis is separated into acute, latent and congenital toxoplasmosis drug therapy. Spiramycin is a drug therapy for toxoplasmosis; used if maternal infection is occurred but the fetus is not infected with parasite (Spiramycin is used to prevent parasite passing through placenta from mother to fetus; which preventing vertical transmission).

Pyrimethamine and Sulfadiazine are used in state the fetus is infected. Pyrimethamine acts as antagonist with Sulfadiazine. The synergistically effect between these two drugs

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results in a significant decrease in disease severity. (Paquet et al. 2013) In immunocompromised patients such as organ transplant recipients, AIDS patient sand malignancies treated with immunosuppressive or cytotoxic drugs. (Gebremedhin 2014)

1.6. Epidemiology of toxoplasmosis:

Toxoplasmosis is found worldwide the approximately 25 to 30% of the worlds human population is infected. (Gangneux and Marde 2012) The geographic distribution to the toxoplasmosis vary widely between one country to another or even within the same country. This variation has been attributed to climate weather, anthropogenic factor including (feeding habits that consist of hand washing, method of cooking meat and vegetable cleaning. (Chao Yan et al. 2016) Toxoplasmosis in animals was studied by sero-diagnosis, and it was found to be 16.10% of 205 tested cattle, 17.65% of tested sheep and 12.09% of 306 tested goats. (Samad et al. 1993) The disease was reported in chicken and cows. Other studies reported the presence of the disease in many neighboring Mediterranean Arab countries, Arabian Gulf countries, Turkish, Beirut, Iran and Jordan. (Khammari, Saghrouni et al. 2013; Jones, Wilson et al. 2001; Montoya, Bennett et al. 2000). The prevalence rate of the disease in Qatar targeting 823 women of childbearing age was found to be 35.1% and 5.2% for *Toxoplasma gondii* IgG and IgM, respectively. (Abu-Madi et al. 2010) Toxoplasmosis in Palestinian cities is considered of an important public health problem and the disease was proven to be endemic in Palestine, based on sero-prevalence studies. In Hebron district; it was found that a total of 27.9% of the examined pregnant women were positive for either IgG and/or IgM antibodies. (Nijem and Al-Amleh 2009) While in Gaza city, the sero-prevalence rate among 312 aborted women was found to be 17.9% based on IgG antibodies and 12.8% based on IgM antibodies titers. (Al-Hindi and Lubbad 2009) Other studies in the region showed that sero-prevalence rate

in reproductive age women was 15.1% in Jews, 25.4% in Bedouins and 72.3% among non-Bedouins Arabs. There is another study in two districts in Northern Palestine (Jenin and Tulkarm districts); the seroprevalence of *Toxoplasma gondii* in goats in Jenin district was 17.44%, while in Tulkarm district was 7.69%. (Othman and Al-Zuheir 2014) In addition, the seroprevalence of *Toxoplasma gondii* in sheep, chicken and turkeys was determined by MAT. This study was showed the seroprevalence of the antibodies for this parasite in 4-6 month old sheep in Beer Sheva (in the south of Palestine); which were 8.9% and 65.6% in older sheep. In a central region, 12% of 100 chickens in a region approximately 45Km north-east of Tel-Aviv and 10.5% of 57 chicken in Jerusalem were be founded seropositive for *Toxoplasma gondii*. The 45 turkeys also in a location about 56Km South east of Tel-Aviv were founded seropositive with antibody titer ≥ 1:144 and 4 of turkeys were founded with antibody titer ≥ 1:50. (Salant et al. 2016)

1.7. Toxoplasma gondii infection: Clinical manifestations and Control method:

1.7.1. Clinical manifestation in human:

Sever disease is usually observed only in immunosuppressed individuals (AIDS) and in children (infected congenitally). (Baron, 1996) Encephalitis is the most clinical manifestation of toxoplasmosis in immunosuppressed patients; which this infection results from reactivation of the tissue cyst in the brain to fast growing tachyzoites. Symptoms of Encephalitis may include disorientation, headache, reflex changes, coma and may lead to death. (Baron 1996; Gebremedhin 2014) On the other hand, Lymphadenitis is the most common manifestation in human that includes fever, malaise, fatigue, sore throat, and headache and muscle pain. (Baron 1996)

Type II genotype is the most cases of congenital toxoplasmosis in Europe and in the United States. (Lindsay and Dubey 2011) *Toxoplasma gondii* leads to a wide spectrum of

clinical disease in children that infected congenitally often infect the retina and brain, whereas severely diseased children exhibit retinochoroiditis, convulsions, intracerebral calcifications and hydrocephalus sings. (Baron, 1996)

1.7.2. Clinical manifestation in animals:

Toxoplasma gondii is not only causing severe diseases in human and has also capability to cause severe disease in animals. *Toxoplasma gondii* infection of animal can be important cause of abortion in goat and sheep in many countries. Cats, dogs and many other pet have pneumonia, encephalitis and hepatitis clinical sings that may lead to death to these pets due to toxoplasmosis. (Baron 1996; Gebremedhin 2014) Abortion, stillbirth and death in neonates clinical sings are observed in pigs. (Gebremedhin 2014) Certain species of avian, marsupials, hares, marsupials, hares and new world monkeys are highly susceptible to fatal toxoplasmosis. (Baron 1996; Gebremedhin 2014)

1.7.3. *Toxoplasma gondii* prevention and control method:

Prevention of Toxoplasmosis is depended on avoiding the ingestion of tissue cysts and oocysts. There are three types of *Toxoplasma gondii* control methods, the first type is primary control method that is included educational materials that contain messages to prevent individuals from becoming infected. Especially, both pregnant women and the women who are becoming pregnant must be educated to avoid infection with *Toxoplasma gondii* through health care policy makers and physicians. (Goldstein, Montoya et al. 2008; Lopes, Goncalves et al. 2007)

At the first visit, health care provides the women and the immunosuppresed persons with the informations that include food hygiene, meat should be cooked (160˚f) and avoiding exposure to cat feces through wearing gloves when they are gardening or

handling soil. (Jones, Wilson et al. 2001; Jones and Dubey 2012) Early diagnosis of the mother, the fetus and the newborn refer to secondary prevention method. Avoiding actions that can lead to parasite transmission through placenta also consisted within the secondary control to toxoplasmosis. (Lopes, Goncalves et al. 2007) Tertiary control method is named serological screening, it is important to identify women who acquire *Toxoplasma gondii* during gestation and also to identify fetal infection through prenatal test. Tertiary controlling focused on early diagnosis to IgM and IgG antibodies in blood samples collected from the newborn. (Goldstein, Montoya et al. 2008; Lopes, Goncalves et al. 2007)

1.8. Vaccination:

During recent years, the research for vaccine to prevent toxoplasmosis was made, but it is still a little to offer against this type of parasite. Here is no vaccine to protect humans against toxoplasmosis. (Lopes, Goncalves et al. 2007) Human vaccination with live mutant or avirulent strains not be recommended because the strains may lead to risk to fetus and may lead to disease, especially in immunosuppressed individuals. (Jones and Dubey 2012) Alive *Toxoplasma gondii* tachyzoites vaccine is used in sheep in Europe and New Zealand. (Innes, Bartley et al. 2009) This vaccine produces protective immunity for 18 months to decrease losing of lambs. (Jones and Dubey 2012) "Toxovax" is a commercial vaccine and alive attenuated S48 strain in ewes and for prevention of ovine abortion, but this vaccine has side effects, a short shelf- life and expensive cost. This vaccine not suitable for human use. (Liu, Singla et al. 2012) In addition, a commercial vaccine that is used to cat no licensed because it has a short shelf life, high cost and must be kept frozen. (Jones and Dubey 2012) Alive-attenuated vaccines are able to activate MHC class 1-restricted CD+ T-Cell response. On the other hand; the crude antigen vaccine, killed vaccines and in

activated vaccines of *Toxoplasma gondii* can not sufficient. DNA vaccine has advantage in that it can elicit humoral and cellular immune responses (CIR). This vaccine generates only weak immune responses when used in humans and high primates. (Liu, Singla et al. 2012)

1.9. Immunity to *Toxoplasma gondii*:

When the *Toxoplasma gondii* infects the cell, the immune responses will be generally developed. Since the *Toxoplasma gondii* is an intracellular parasite, the infection is controlled by T-cell mediated immune responses and ability of CIR in resistance *Toxoplasma gondii* infection determine the survival of the host, but when the immune responses not able to be elicted or stimulated, the parasite will invade the host and lead to toxoplasmosis; which there is balance between the survival of parasite and the host cell. (Denkers and Gazzinelli 1998; Taweenan 2004)

Capacity of *Toxoplasma gondii* to spread in all the tissues of the host cell and the capacity of the recurrent infection with new strains make the immune responses against *Toxoplasma gondii* more complex. (Filisetti and Candolfi 2004) Many studies revealed that the *Toxoplasma gondii* infection controlling need pro-inflammatory production of cytokine IL-12 that stimulates CD4$^+$, CD8$^+$-Tcells and Natural Killer (NK) to IFN-ɣ (gamma interferon). (Hunter and Sibly 2012; Johnson 1992; Gazzinelli et al. 1993) IFN-ɣ and tumor necrosis factor alpha (TNF-alpha) cytokines have ability to activate macrophage; which both cytokines are important in tachyzoite replication controlling during acute and chronic infection. (Sibley et al. 1991) IL-12 appears more important during initial infection and less important during chronic infection of *Toxoplasma gondii*. IL-12 plays a crucial role in activation CMI against *Toxoplasma gondii*. (Denkers and Gazzinelli 1998; Gazzinelli et al. 1994)

Invasion of *Toxoplasma gondii* into host cell activates the innate compartment of immune response such as macrophages and NK cells. (Denkers and Gazzinelli 1998; Taweenan 2004) Macrophages play effector roles; which phagocytic cells contain on their surface antibodies that act as opsonion, these antibodies bind with *toxoplasma* antigens through Fab regions and bind with phagocytic cell (macrophage) through Fc region. Dead or opsonized parasites are degraded by lysosomal compartments, whereas live parasites that invade the host cell are established in protective parasitophorous vacuole (PV). (Hunter and Masek 2000; Mordue and 1997; Sibley, Weidner and Krahenbuhl 1985) Expansion of NK cell is based on $CD4^+$ Lymphocytes, through the production of IL-2 itself. NK cells classified as a major source of IFN-ɣ.

Earlier studies revealed that the murine NK cells have ability to produce IFN-gamma when it is stimulated through co-stimulatory molecules as CD28. (Denkers and Gazzinelli 1998) Several studies indicate that the antibodies also play a crucial role in immunity against *Toxoplasma gondii* beside the CMI responses, such as antibodies that are specific to SAG1 inhibit the invasion of tachyzoites into human fibroblast cells. (Mineo, Mcleod et al. 1993; Taweenan 2004)

1.10. Antigens of *Toxoplasma gondii*:

The first component of *Toxoplasma gondii* that binds with the host cells is the surface antigen of parasite. (Liu, Zhang et al. 2006) Many previous studies have revealed that the SAG1 is the most important ligand that binds with the host cell and is the major surface protein to the *Toxoplasma gondii* parasite. (Robinson, Smith et al. 2004) Surface antigens are playing essential role in neutralizing antibodies and CMI responses, such as SAG1 and SAG2 A that stimulate the humoral immune response in the acute infection. (Partanen, Turunen 1984; Séguéla, Bessieres et al. 1992) SAG1 surface protein also

stimulates T-cells that are producing IFN-gamma. (Khan, Eckel et al. 1988; Kim and Boothroyd 2005) Tachyzoite-specific antigens such as SAG1 and SAG2A and Bradyzoite-specific antigens can activate an immune respons. (Kim and Boothroyd 2005) In humans suffering from chronic infection of Toxoplasmosis, the immune responses against bradyzoite-specific antigens, such as SAG2C/D (which is a member of SRS family) and SAG4, LDH2, ENO1 and P-ATPase are undetectable. (Di Cristina, Del Porto et al. 2004)

Apicomplexa parasites are able to invade host cells by unique mechanism involving secretion of secretory vesicles called transmembrane micronemal proteins (TM-MICS). TM- MICS play a role in host cell attachment, gliding motility, rhoptry secretion and invasion (Sheiner, Santos et al. 2010) In addition, TM-MICS provide the connection between external receptors and the submembranous acto-myosin motor that provides power for parasite gliding and host cell invasion. (Cérède, Dubremetz et al. 2005)

The other surface protein is Rhoptries that are essential in *Toxoplasma gondii* penetration and interaction of host cell. Most ROP proteins are Apicomplexa- specific proteins and some of them contain enzyme signature, they are important in organelle function and biogenesis. (Dubremetz 2007) *Toxoplasma gondii* parasits have bulb of the rhoptry organelle that releases ROP protein when the parasite invade the host cell. (Hunter and Sibly 2012) According to previous studies, ROP proteins are discharged and attached to host cell during invasion, resulting in the prediction of effector proteins that alter the signals of the host cell. (Weiss and Kim 2007) When the ROP protein invade the host cell it can activate the signal transcription and transduction pathways (STAT) to stimulate the expression of cytokines and ROP protein also binds and inactivates PV membrane. (Denkers, Schneider et al. 2012; Martens, Parvanova et al. 2005)

Dense granule proteins (GRAs) is also a soluble proteins released from dense granules into the PV and have two groups; the first group consists of proteins that lack homology to

organism and the second group consists of proteins that have homology in other Eukaryotes. (Rome, Beck et al. 2008) All the GRA proteins are expressed as excretory/secretory antigens and composed of 25-30 amino acids except GRA3 and GRA6 antigens; GRA3 composed of 22 amino acid and GRA6 antigen contains 23 amino acids. (Hung, Falcone 1996; Nam 2009) GRA proteins of *Toxoplasma gondii* parasite stimulate the maintenance of host- parasite interactions across the PV membrane.

1.11. Random Phage Display Libraries Technology (RPDL):

Phage Display libraries is a technology for identification of epitopes or protein that mimic protein or non-protein epitopes and protein discovery. (Smith and Petrenko 1997; Wang and Yu M 2004) Applications of phage display technique are several including diagnostic, epitope discovery, enzyme specificity and inhibitors, agonists, antiagonists identification to probe, the receptor structure and function, protein-protein interactions and target specific antibodies generation which can be useful in specific drug delivery (vaccination development). (Wang and Yu M 2004)

Phage display is considered as the powerful methodology that includes production and screening a large number of random peptides of a specific length (8-20 amino acids) that are expressed on the surface of phages (Devlin et al. 1990) accordingly, antibodies react with peptides and proteins displayed on the surface of filamentous bacteriophage. (Morozova)

Initially, in 1985, George Smith performed this technology, Smith demonstrated that peptides on the surface of M13 bacteriophage can be expressed and affinity enrichment method termed "Biopanning" was developed in 1988. (Parmley and Smith 1988) Biopanning is the most common screening method that is based on phage clone enrichment with binding affinity for the target. Biopanning process involves immobilization of target

molecules to 96- well microtiter plates, phage binding; which the RPDL is added to the target coated well. Removing unbound phage, phage elution (elution of the target bound phage can be carried out in a solution containing free target or competing ligand) and amplification of eluted phage, repeating biopanning process three to six times, and analyzing the phage clones by DNA sequencing to identify the epitopes that bind with target (Pande and Szewczyk 2010). After bacterial-based amplification, the phagemids is collected and the relevant DNA within the interacting phage is sequenced to select the interacting protein fragments or interacting epitopes.

There are many phage display systems; the most common phage display system is RPDL. Success of phage display technology defined by the properties of filamentous bacteriophages. These bacteriophages infect gram-negative bacteria (Morozova and Tikunova 2009) by attaching F-pili, all coat proteins are inserted into the inner membrane of Escherichia coli bacteria before integrated into the phage particles (Jacobsson, Rosander et al. 2003). The best characterized are the M13, f1 and fd phages that infect E. coli strains that carry F- conjugative plasmid. (Morozova and Tikunova 2009) The genomes of f1, M13 and fd filamentous bacteriophages are more than 98% identical and their gene products interchangeable. (Pakonjac, Bennett et al. 2011) Filamentous phages have many features including a fixed diameter , and about 1μm long, the phage contains a circular single strand DNA genome that wrapped inside several copies of pv III major coat protein. (Wang and Yu M 2004) Phage particles are consist of five coat proteins; which are p III, pv III, pv I, p IX and pv II. These coat proteins are found on the surface of bacteriophage. (Pakonjac, Bennett et al. 2011)

In this study; the Ph.D. [TM] system or Ph.D-12 Random Peptide Phage Library (New England Bio Labs, Beverly, MA) was used; this system based on a simple M13 bacteriophage vector that expresses 12 different amino acids. M13 phage libraries

commercially available, which present peptides at the N-terminus of the phage P III coat protein (these peptides displayed at one end of the filamentous phage in 3-5 copies. (Gray and Brown 2013)

One phage display technique application is cloning of DNA encoding ligand-binding domains of prokaryotic receptors from chromosomal DNA. This technique in identification of receptor gene needs for probes, so it can be used for cloning prokaryotic receptor genes without prior knowledge of the receptor. (Jacobsson and Frykberg 1996) Phage display technique is used to obtain a macromolecule proteins (peptides) that mimic the structure of an epitopes. These macromolecule proteins are named mimotopes. (Editorial 2001; Phalipon, Folgori et al. 1997)

1.12. Objectives:

The overall aim of this study is to identify immunoreactive *Toxoplasma gondii* epitopes using random phage display library screened by human positive sera of high antibody titers against *Toxoplasma gondii*. The specific objectives are:

1. Identification of high anti-*Toxoplasma* sera by modified agglutination test (MAT).

2. Screening of phage display peptide library by bio-panning method.

3. Validation specific interaction between the selected epitopes and anti-*Toxoplasma* sera by adapting Dot-ELISA and ELISA.

4. Characterization of selected epitopes by PCR, DNA sequencing and BLAST analysis.

Part 2: Materials and Methods.

2.1. Serum Collection:

Serum samples were collected from pregnant women attending the two main Hospitals in Hebron district; namely (Al-Ahli hospital in Hebron and Yatta Hospital). In the last year (2015), an official cooperation was established with the Hospitals administrations in order to organize sample collection. The total number of collected samples were 204 samples.

2.2. Toxoplasmosis Serodiagnosis Screening by MAT:

The 204 collected sera samples were screened for the presence of *Toxoplasma gondii* infection based on serological examination for collected sera. Initially, the simple Modified Agglutination Test (MAT), was used for direct screening and evaluation for the presence of IgG antibody against *Toxoplasma gondii* parasite. The MAT was conducted in 96-well-U-bottomed microtiter plates. The tested sera were diluted in phosphate buffer saline (PBS) (1.8 mM KH_2PO_4, 150mM NaCl and 10.1mM Na_2HPO_4). *Toxoplasma* cells were prepared by mixing 150µl formalized *Toxoplasma gondii* tachyzoites (Reims, France), 3.5ml antigen buffer (phosphate buffer tween), 50µl of Evans blue stain and 35µl of 2-mercaptoethanol. Some Sera were diluted to (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320) for MAT testing; In each (A, B, C, D, E, F, G, H) well of microtiter plate; 90µl of serum diluting buffer (PBS) and 10µl of serum were added. In other wells (2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) 50µl of antibody diluting buffer was added. 50µl of each diluted serum was transferred to make serial dilution. In each well of microtiter plate 50µl of *Toxoplasma gondii* antigen was mixed with antibody diluting buffer. Others sera were diluted (1:5 and1:10) for screening the presence of *toxoplasma* infection. Negative control sera and positive control sera were included. The 96-well-U-bottomed microtiter was covered and incubated at room temperature for 24 hour. The collected sera samples finally were screened for the presence of *Toxoplasma* infection after incubation period based on

agglutination (positive result) or concentration of formalin fixed tachyzoites in the well bottom (negative result). (Liu, Wang et al. 2015)

2.3. Antigen Preparation for immunological studies:

Toxoplasma gondii Antigen was prepared for SDS-PAGE Gel Electrophoresis and Western Blot analysis according to the following procedure: 100μl formalin fixed tachyzoite of *Toxoplasma gondii* (Reims, France) was mixed with 100μl lysis buffer of cells (10mM EDTA, 50mM Tris, 50mM NaCl, 0.01% SDS and PMSF Protease inhibitors (Sigma, USA)). Toxo- cells and lysis buffer were incubated 30 min at 60°C and kept at − 20°C for 15 min. Then incubated for 30 min at 60°C. Finally, it was centrifuged for 10 min. *Toxoplasma gondii* parasite lysates containing crude antigen were frozen; crude antigen was thawed prior each use for SDS-PAGE Gel Electrophoresis and Western blot.

2.4. SDS-PAGE Gel Electrophoresis (SDS-PAGE):

Firstly, 10%polyacrylamide resolving gel (1.5M Tris-HCL, pH8.8) and 5% polyacrylamide stacking gel (0.5 M Tris-HCL, PH6.8) were prepared. A crude antigen lysates of *Toxoplasma gondii* (protein samples) and selected amplified phages were boiled in water bath with loading buffer or reducing sample buffer (2.5ml glycerol, 0.2ml 0.5% Bromophenol Blue, 50μl 2-mercaptoethanol, 2.0ml 10% SDS, 1.25ml 0.5M Tris PH6.8 and 3.55ml deionized water) for 10 min and separated in a SDS-Tris-glycine buffer through polyacrylamide gel electrophoresis (PAGE). 10μl of marker, antigen lysates-loading buffer mixture and selected amplified phages were loaded on the SDS-PAGE Gel. The stacking gel was run at 50 volte (V) (20mA) and running of resolving gel (Separating gel) was done at 100V. Broad range Bio-Rad molecular weight markers (Land Mark ᵀᴹ Prestained Protein Marker, Mbiotech) were used. (Yang and Hongbao 2009)

2.5. Coomassie Brilliant Blue Staining:

After polyacrylamide gels were run, the gels were stained with coomassie brilliant blue stain (1g coomassie blue R250, 225ml methanol, 225ml acetic acid and 100ml distilled water) for 15 min and de-stained with de-staining solution (400 ml D.W, 50ml methanol and 50ml acetic acid).

2.6. Western Blot Technique (Protein Immunoblotting):

Analysis by western blot was done according to Harry Towbin, 1979. (Towbin, Staehelin et al. 1979) This technique involves transfer of proteins from the polyacrylamide gel onto nitrocellulose membrane (Whatman, USA). The gel was equilibrated in 1X transfer buffer (14g glycine, 3g tris-base, 100ml methanol, 900ml double distilled water) for 1 hour on 100V. The proteins were transferred from gel to nitrocellulose membrane (Whatman, USA). After transfer, the membrane was cut into strips and blocked with blocking buffer (100ml phosphate buffer saline-Tween-20 (PBS-T) and 5ml Fetal calf serum (FCS)) for 30 min at room temperature (RT). Strips then were incubated for 2 hours at RT with shaking on shaker with diluted 1:100 and 1:200 *Toxoplasma gondii* high antibody titer pooled sera. Unbound antibodies were washed 3 times for 5 min with 3ml PBS-T. After that; secondary antibodies (protein A–HRP) diluted in PBS-T (1:6,000) for 1 hour with shaking at RT. Excess secondary antibodies were washed off 3 times with PBS-T. Bound antibodies were detected with DAB substrate (10mg DAB, H_2O_2, 15ml 50Mm Tis-Hcl). Strips were incubated for 15-30 min at RT until band color was observed. Also bound antibodies were detected with Enhanced Chemiluminescence (ECL) and according to the manufacturer instructions (ThermoFisher Scientific, USA).

2.7. Ponceou-S Stainning:

After transfer, the membrane was submerged in Ponceou-S stain for two min. Ponceou-S solution (0.1% Ponceou-S, 50% glacial acetic acid in aqueous solution) which can be reused.

2.8. Phage Display Peptide Library:

The Ph.D.^{TM} Phage Display Library Kit # E8110S (New England Biolabs, Ipswich, MA, USA) was used according to the manufacture recommendation with some alterations as following:

- **Lauria broth (LB) Media:** LB media was prepared by dissolving 10g peptone, 5g yeast extract and 5g NaCl in 1L double distilled water, then autoclaved and stored at 4°C until used.

- **LB Plates:** 35g LB Agar (Difco^{TM}LB Agar, Lennox) was dissolved in 1L double distilled water, autoclaved for 15 minutes and then poured into petri-dishes. Plates finally were stored inverted at 4°C until used.

- **LB Top Agar:** per 250ml: 1.75g yeast extract, 2.5g Nacl and 2.5g peptone. Double distilled water then was added up to 230ml and the solution was dissolved. After that, 1.8g agar was added, autoclaved and stored at 4°C until used. The Top agar was melted in microwave and equilibrated at 50°C before use.

- **Bacteria:** *E. coli* ER2738 host strain was used. 5ml LB Broth was inoculated with *E. coli* ER2738 bacteria and incubated at 37°C for overnight with shaking.

- **IPTG\ X-gal:** 1M IPTG (Isopropyl-Beta-D-thiogalactoside) was prepared (2.3g IPTG was dissolved with 10ml D.D.H$_2$O). X-gal (5-Bromo-4-chloro-3-indolyl-beta-D-galactoside) also was prepared (0.2g X-gal was dissolved in 10ml DMSO (Dimethyl sulfoxide). X-gal was stored at -20°C in the dark.

- **TBST (Tris-bufferd saline and Tween 20):** To 500ml TBST, (25ml Tris 7.5, 1.5ml 5M NaCl, 250 μl Tween-20 and 500ml D.D.H2O.

2.8.1. Surface Panning using anti-*Toxoplasma* reactive pool sera:

2.8.1.1. Binding of reactive phage epitopes:

A solution of the pooled sera in 0.1M Sodium bi-carbonate (NaHCO3) (1.05g NaHCO3 dissolved in 100ml D.H2O) was prepared. 100μl of this solution was added to each well of 96-well microtiter plate or 500μl of this solution was added to each well of 24-well plate. To coat plate with pooled sera, the plates were incubated overnight at 4˚C. In the second day; the coating solution from each plate was firmly slapped face down onto a clear paper towel. Next, each well of plate was filled with blocking buffer (100ml TBST and 5ml FCS) and incubated for one hour. After that, the blocking buffer was discarded and each plate was washed 3 times with TBST. M13 phage vector\library then was added onto each plate after the dilution with TBST was done and incubated for one hour at room temperature. After that, nonbinding phage was discarded by pouring off and slapping plate face down onto a clean tissue paper. Each plate was washed 10 times with TBST. Bound phage finally was eluted with glycine elution buffer (0.75g glycine was dissolved with 50ml D.D.H2O) and the elution buffer was rocked gently for no more than 10-20 minutes. The eluted phage was amplified as described below.

2.8.1.2. M13 Bacteriophage Amplification:

The eluted phage was amplified by adding into 5ml ER2738 culture (early-log/ 5ml Lb broth and 50μl bacteria) and incubated with vigorous shaking at 37˚C for overnight. After incubation period, the culture was transferred to centrifuge tube and centrifuged for 10 minutes at 14,000rpm. Next, 1ml supernatant was transferred to a new fresh tube and 200μl of 20%PEG/2.5M NaCl was added. Phage was allowed to precipitate for overnight

at 4˚C. The PEG precipitation then was spun at 14,000 rpm at 4˚C for 10 minutes and the supernatant was discarded. The phage pellet was suspended with 1 ml TBS, 100μl of 20% PEG/ 2.5M NaCl was added. Then it was incubated at 4˚C for 1 hour. After incubation period, the tube was centrifuged at 14,000rpm for 10 min and the supernatant was discarded and 100μl TBS was added onto the phage pellet; which at this step the amplified phage was obtained (first round of panning was carried out). The Amplified phage was used in a second M13 phage surface binding/screening, and the amplified phage of the second screening round was used in a third round of screening. The M13 phage that was produced from the third screening round (surface binding) was amplified and named as the final amplified phage. This bio-panning process was done at least five times and in each time the third amplified phage was collected. The resulted amplified phage was tittered on LB/IPTG/X-gal plates. (section 2.8.1.3.)

2.8.1.3. M13 Bacteriophage Titration:

The number of plaques was linearly increased with added phage obtained from phage Display library Kit) when the bacterial cells were in excess (multiplicity of infection (MOI) was much less than 1). 5ml broth was inoculated with ER2738 from a freshly grown culture and incubated for overnight at 37^oC until had reached mid-log phase, (reach OD_{600} of about 0.5). In the next day, top agar was melted in microwave and dispensed into 5ml in 15ml sterile culture tubes. Each tube was maintained at 50^oC. Serial dilutions of M13 Bacteriophage in LB (10^3 to 10^{16}-fold) were prepared in 1ml LB final volume. After the ER2738 culture had reached mid-log phase, 200μl were dispensed into microfuge tubes, one for each phage dilution. 200μl ER2738 bacteria and 10μl phage then were added into top agar. Tubes were briefly and immediately poured onto a pre-warmed LB/IPTG/X-gal

plate. Plates were then gently rotated to spread top agar evenly. Plates were incubated overnight at 37°C.

2.8.2. M13 phage plaque assay:

After performing phage titration test, it was found that the phage titer is up to 10^{15}; this titer produced about 100 separated plaques per plate. The final amplified M13 phage was grown on 25 different plates in a dilution of 10^{12} (pfu/ml), the obtained plaques from each plate were transferred into nitrocellulose membrane (Whatman, USA) and then screened to their reactivity against anti-*Toxoplasma gondii* pooled sera as described below.

2.8.3. Plaque lifts into nitrocellulose membranes:

Before the plaques were lifted onto nitrocellulose membrane, the prepared M13 phage plates were kept for overnight at 4°C to prevent the top agar from sticking to the nitrocellulose membrane and increase titer\plaques. To each LB agar/IPTG/X-gal plate containing plaques a nitrocellulose membrane was placed over the plaques for 5 min to allow the transfer of the phage plaques to the membrane. A prick through the membrane and agar was done by a pen for orientation. The plates then were incubated for 30 min at 37°C and then incubated for 1 hour at 4°C. The membranes next were removed from the plates and kept at room temperature for overnight to dry and to fix the phage protein to the membrane. The dried membranes were submerged in phosphate buffer saline for few minutes and then blocked with 5% FCS in PBS with Tween-20 for 30 min. Finally, screened by anti-*Toxoplasma gondii* high antibody titer positive human sera as mentioned in Western blotting. (See 2.6.)

2.8.4. Selected Plaques screening by *anti-Toxoplasma* Pooled Sera:

All selected plaques that showed reactivity with *Toxoplasma gondii* pool sera (High Antibody titer) were picked by sterile micropipette tips and used to inoculate 1.5ml tubes

containing 300µl LB medium. All tubes were kept at 4°C. Reactive plaques with high Antibody titer human pooled sera (Selected plaques) were amplified as mentioned in phage amplification (see 2.8.1.2) to suspended and obtained precipitate phage to perform Dot-ELISA (Phage Dot-Blot Assay) and phage ELISA binding assay. The phage dot-blot assay (Dot-ELISA) and phage ELISA binding assay were done as described below.

2.9. Dot-ELISA (Phage Dot-Blot Assay):

A total of selected M13 phage clones (amplified reactive plaques) suspended with TBS were prepared and concentrated (100µl, 50µl and10µl) and (30µl and 10µl). Nitrocellulose membrane were soaked in 1x PBS and placed on dot blotter apparatus (Bio-rad, USA) which was connected to vaccume pump. These selected M13 peptide phage clones were blotted onto the nitrocellulose membrane and allowed to air-dry for overnight at room temperature, the membrane then was blocked with 5% FCS and 100ml PBST for 30 min at room temperature (RT). Membrane was incubated for 2 hours at RT with shaking in diluted 1:500 *Toxoplasma gondii* high antibody titer pooled sera. Unbound antibodies were washed 3 times for 5 minutes with PBS-T. Secondary antibodies (protein A–HRP) diluted in PBS-T (1:6,000) was added for 1 hour at RT. Excess secondary antibodies were washed off 3 times with PBS-T. Bound antibodies were detected with ECL (Film-developer). (Sastry, Tuteja et al. 2003)

2.10. Enzyme Linked Immunosorbent Assay (ELISA):

Two microtiter plates were coated with isolate and concentrated phage (amplified phage) in duplicates, they incubated for 24 hours at 4°C and then blocked (100µl/well) with 100ml PBS-T and 5%FCS and incubated for 40 minutes at room temperature and washed 3 times with PBS-T. Next, 100µl of 1:200 serially diluted pooled sera were added as first

Abs and incubated for 2 hours and washed 3 times with PBS-T. 100µl diluted protein A (1:6,000) was added as second antibodies and incubated for 1 hour at room temperature then washed 3 times with PBS-T. 200µl of the substrate solution (40mg of the O-phenyldiamine (OPD), 30ml of 0.1M tri-sodium citrate buffer (pH5)) and 30µl of H_2O_2) was added and incubated for 30 min at room temperature. Yellow color development finally was measured at 490nm using ELISA auto-reader. Amplified phages that showed high antibody titer were coated again into 96-well microtiter plates. Five amplified phages were chosen to be tested if there are reactivity between them and sera of *Toxoplasma gondii* infected individuals. (Jalallou, Bandepour et al. 2010)

2.11. Polymerase Chain Reaction (PCR):

All selected phage clones were detected by phage dot-blot (Dot-ELISA). These selected clones were identified by amplification reactive phage gene by Polymerase Chain Reaction (PCR). PCR analysis was done by using primers against a portion of the M13 Bacteriophage gene. The Ph Rev2 primer and Ph Direct2 primer (Sigma Chemical Co.) were used. PCR analysis firstly was carried out for 27 samples (including the negative controls), a master mix tube was prepared as followed: 540µl double distilled water, 30µl of 20 pmoles of ph Direct2 primer and 30 ph Rev2 primer, then it was mixed well and 20µl was dispensed in each Ready Mix PCR tubes (Syntezza, Jerusalem) followed by the addition of 5µl from each extracted DNA sample. The used thermal profile in thermo-cycler involving 5 min at 95°C to initial denaturation, followed by 35 cycles each of 30 second at 95°C to complete denaturation, 30 sec at 55°C TM (depends on the used primers) to perform annealing, followed by extension step (elongation) that involved 1 min at 72°C, and a final elongation step at 72°C for 10 min. PCR analysis of other samples with the same procedure was performed with all 27 samples. (Bourdin, Busse et al. 2014)

2.12. Agarose Gel Electrophoreses:

PCR products were run on a 1.5% agarose gel (1.5g agarose, 100ml 50X TAE and 10µl Ethidium bromide in 50X TAE electrophoresis running buffer (242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (Ph8.0)). The Gene Ruler 50bp DNA ladder (Thermo Scientific, # SM0371) was used for sizing PCR amplified products. (Ramberg 2006)

2.13. DNA Purification:

PCR products were purified by GeneJET PCR purification Kit (Thermo Scentific, USA), according to manufacturer's instructions with some modifications. The DNA purification steps were done as following: The remaining quantity of PCR product for each sample was added into eppendorf tube; 100µl of sterile D.D.H2O then was added. Next, 200µl of binding buffer was added in each tube and centrifuged for 60 sec to discard flow-through. 700µl wash buffer (ethanol) was added to the GeneJET purification column for each sample. Then each tube was centrifuged for 3 min and the flow-through was discarded. GeneJET purification column was centrifuged again to remove any residual wash buffer. The GeneJET purification column next was transferred into 1.5ml microcentrifuge tube and 30µl D.D.H_2O was added to concentrate DNA. Then, all tubes were incubated at RT for 2 min and then centrifuged for 60 sec. Then the purified DNA was stored at -20°C; which all samples were ready for sequencing.

2.14. DNA sequencing:

Purified PCR products were sequenced according to dye terminator method, using Automated DNA Sequencer machine (AB477). The primers that used to DNA amplification were used there, for each PCR product primer was used in DNA sequences acquaint.

Part 3: Results.

3.1. Modified Agglutination Test (MAT):

The collected sera samples from pregnant women were screened for the presence of *Toxoplasma gondii* infection. The reactivity of these collected sera to *Toxoplasma gondii* and evaluation of the presence of IgG antibody against *Toxoplasma* parasite were determined based on serological examination for collected sera by simple Modified Agglutination Test (MAT). This MAT test depends on agglutination of formalin fixed *T. gondii* tachyzoite parasite cells. The design of *Toxoplasma gondii* agglutination test and representative results of *Toxoplasma* MAT test in microtiter plate are shown in figures 3.1.1 and 3.1.2.

The antibody titers for the different tested sera samples were identified starting from 1:5 dilution up to 1:320 serum dilution (As shown in Table 3.1.1). The total number of collected sera samples was 204, from which a total of 116 samples gave positive results. The percentage of the tested samples that gave antibody titer of 1:20 and above was 16% from the total, while those that gave 1:5 and 1:10 antibody titer by MAT test was 22% and 19%, respectively (Figure 3.1.3). All positive sera that gave 1:20 antibody titer in MAT test were collected in one pool and was named as (high antibody titer positive human sera), a total of 45ml was pooled.
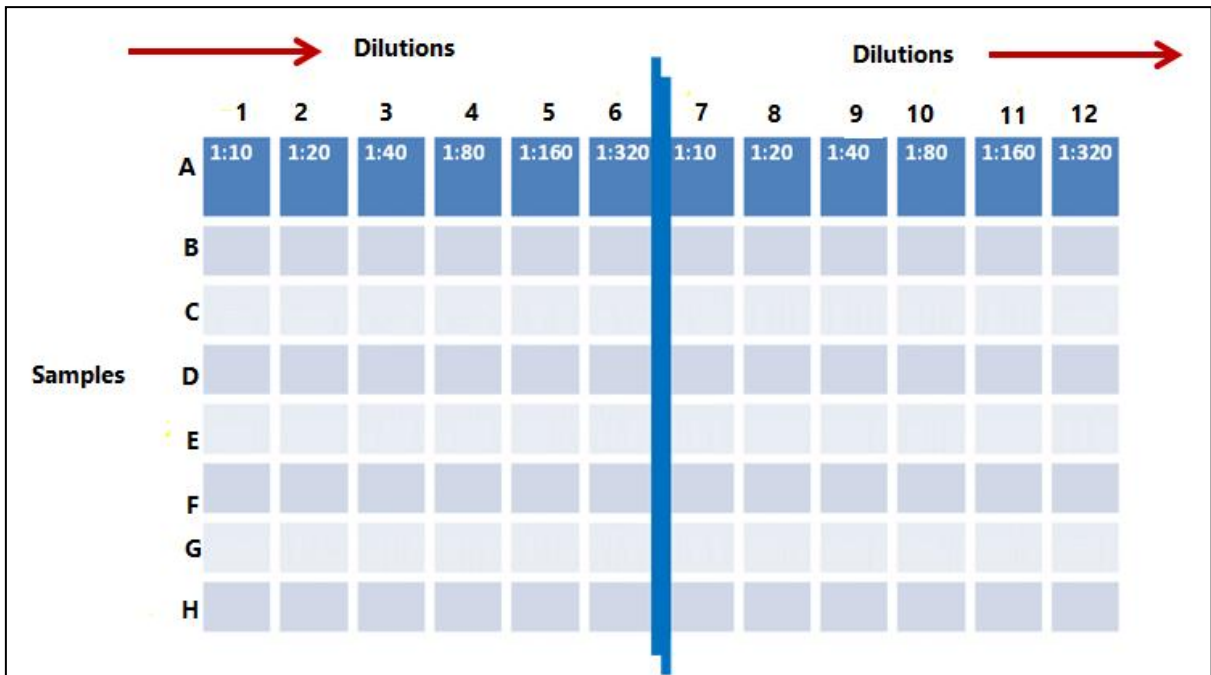
Figure 3.1.1: Plate design of *Toxoplasma gondii* agglutination test. A1-A6: represents positive controls. B1-B6 up to HI-H6 and A7-A12 up to H7-H12: represent the collected samples. A total of 15 samples were tested in this microtiter plate.
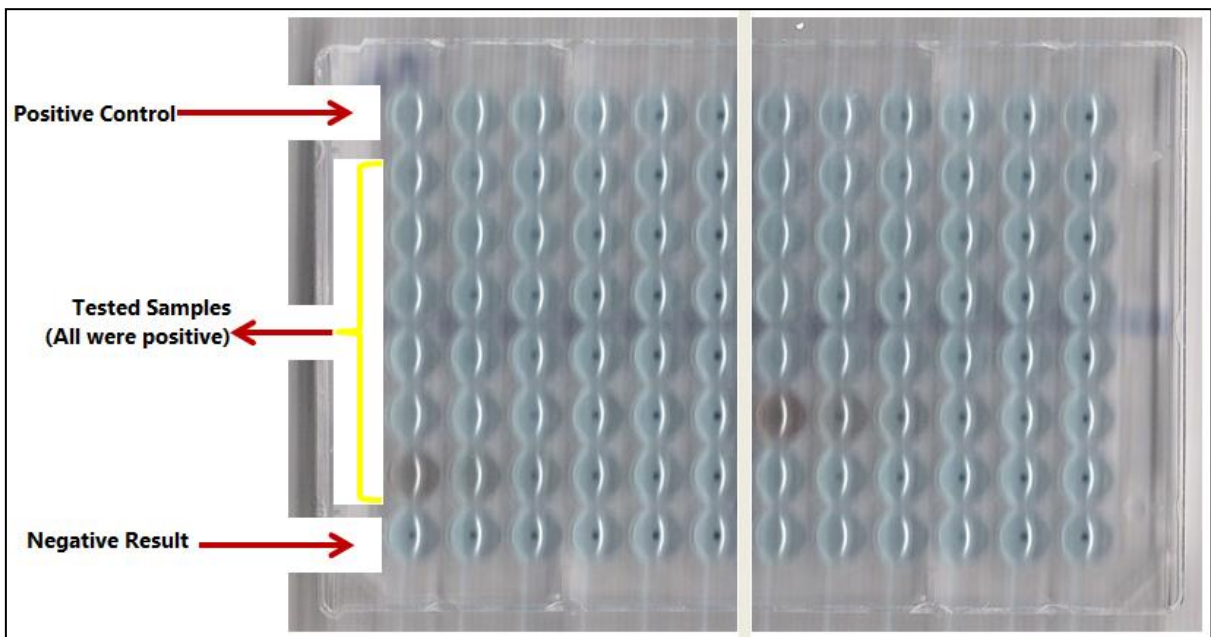
Figure 3.1.2: A representative results of *Toxoplasma* agglutination test. Representing detection of anti-*Toxoplasma gondii* antibodies for the collected sera samples. The collected sera samples were diluted to (1:10, 1:20, 1:40, 1:80, 1:160 and 1:320).

Table 3.1.1: Antibody titers of the different tested sera samples based on
MAT test against formalin fixed tachyzoite *Toxoplasma gondii* parasite.

Antibody Titer	Number of positive samples	Percentage of antibody titer
1:5	45	22%
1:10	39	19%
1:20	12	6%
1:40	12	6%
1:80	3	1.5%
1:160	2	1 %
1:320	3	1.5%
Total number of positives	116	57%
Total number of negatives	88	43%
Total number of tested	204	100%
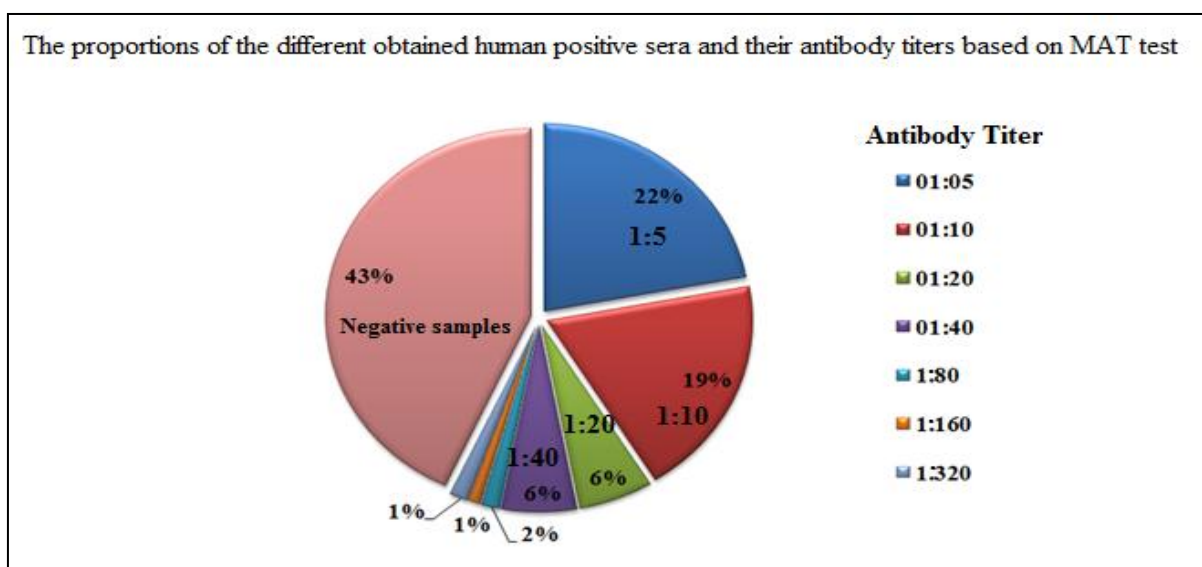
Figure 3.1.3: Pie chart that summarizing the proportions of the different obtained human positive sera and their antibody titers based on MAT test.

3.2. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis:

3.2.1. SDS-PAGE:

Toxoplasma gondii crude antigen extract and amplified phage peptides (resulted from the third round of phage panning) were analyzed by SDS-PAGE and Western blotting techniques. From the prepared extracts; 15µl total volume were run on SDS-PAGE. Four different gels were prepared each containing parasite crude extracts, amplified phage peptides and MW marker. Two of the gels were stained with Commasie brilliant blue stain (data shown only for one gel; Figure 3.2.1). The SDS-PAGE analysis of the *Toxoplasma gondii* crude antigen extract shows a major multi-band that had different molecular weight sizes ranging from 70-100 kDa. The phage peptides showed a major band that had a molecular weight of 60 kDa.
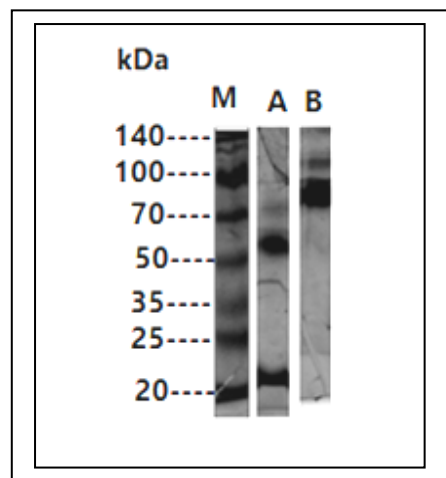
Figure 3.2.1: Commassie Brillient Blue-Stain of 10% SDS-PAGE gel. A: represents amplified phage proteins. B: represents crude extract protein banding pattern. M: Molecular weight marker.

3.2.2. Western blot analysis:

The reactivity of *Toxoplasma gondii* crude antigen extracts with high antibody titer positive human pooled sera collected from *Toxoplasma gondii* infected pregnant women was determined by immuno-blot analysis. The reactivity of the amplified phage (produced from third-round-panning) against high antibody titer positive human pooled sera is shown in figure 3.2.2 and 3.2.3. Immunoblotting analysis revealed one band at molecular weight of 50 kDa using the phage extract and more than one band upon the use of *Toxoplasma* crude antigen extract; with a major band that had a molecular weight of 50 kDa and other minor bands of about 100 kDa (Figure 3.2.2). Similar results were obtained even if more sensitive analysis by the aid of enhanced chemo-luminescence (ECL) assay (As shown in figure 3.2.3). The reactive antibody with *Toxoplasma* crude antigen extract was IgG, since the anti-human IgG-HRP and protein-A-HRP were used as secondary.
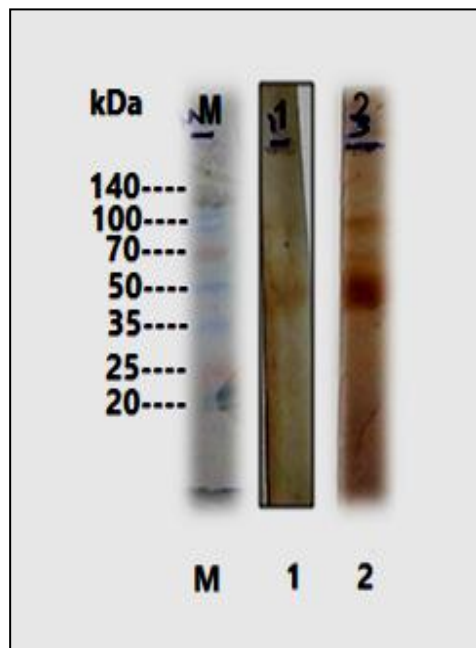
Figure 3.2.2: Western-blot analysis for *Toxoplasma gondii* crude antigen extract and amplified phage using OPD substrate. Representing the reactivity of *Toxoplasma gondii* crude antigen extracts with high antibody titer positive human pooled sera. Protein A-HRP was used as secondary antibody. Strip no.1 represents reactivity of amplified phage with high antibody pooled sera. Lane no.2 represents the reactivity of crude antigen extract. MW: molecular weight
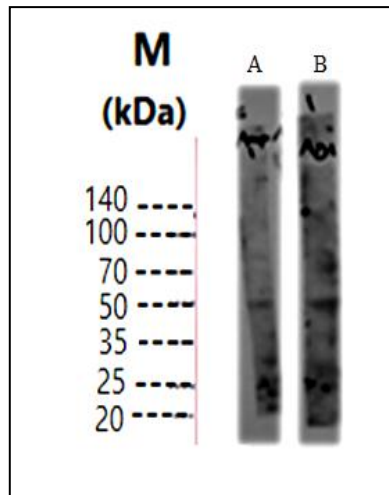
Figure 3.2.3: Western-blot analysis of *Toxoplasma gondii* crude antigen extract and amplified phage detected by highly sensitive enhanced chemo-luminescence (ECL) assay. Representing the reactivity of *Toxoplasma gondii* crude antigen extracts with high antibody titer positive human pooled sera collected from pregnant women. Protein A-HRP was used as secondary antibody. Strip no.A: represents reactivity of amplified phage with high antibody pooled sera. Strip no.B: represents the reactivity of crude antigen extract. MW: molecular weight marker.

3.3. Screening of M13 random phage display library:

The M13 phage library titer indicated by the phage library manufacturer (New England Biolabs, Ipswich, MA, USA), was estimated to be 10^{13} plaque forming units (pfu)/ml. The titer of the phage library decreases along storage time, for this reason and for obtaining a workable number of plaques (separated plaques) in each plate; the phage library was titrated using different dilutions of phage library starting from 10^3 down to 10^{16}. Each dilution was used to infect 200µl of freshly grown *E. coli* bacteria followed by plating on LB agar plates that include IPTG/X-gal as described in materials and methods. After performing phage titration test, it was found that the phage titer was up to 10^{15}(pfu)/ml.

3.3.1. M13 phage screening and selection using anti-*Toxoplasma gondii* pooled sera:

The final amplified M13 phage was grown on 25 different plates in a dilution of 10^{12} (pfu/ml), the obtained plaques from each plate were transferred into NC membrane

and then screened to their reactivity against anti-*Toxoplasma gondii* pooled sera. Plaques that were lift on the NC membrane from each plate was screened by 1:500 diluted anti-*Toxoplasma gondii* pooled sera as first antibody and 1:5000 diluted protein A-HRP or anti-human IgG-HRP as a second antibody (Figure 3.3.1). The highly reactive plaques (plaques that gave strong color signals) were transferred and incubated in 300µl LB media for further analysis. The total number of the obtained M13 reactive phages was 329 plaques from a total of 25 plates (Table 3.3.1), that are estimated to have at least 5000 plaques (assuming in each plate there are only 200 plaques). From the total number of the obtained M13 reactive phages (329 plaques); 138 reactive plaques were picked up and used for further analysis in this study.
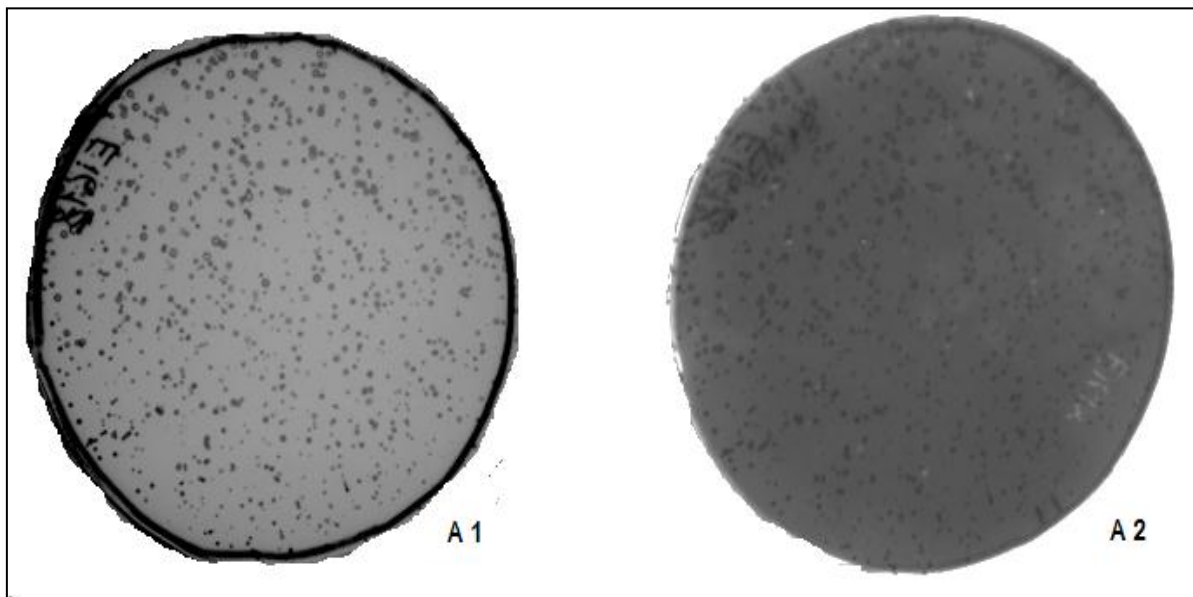
Figure 3.3.1: Plaques Selection and Screening with *Toxoplasma* high antibody titer pooled sera by biopanning process. Panel A1: represents separated plaques when amplified phage tittered on LB agar/IPTG/X-gal plate and Panel A2: represents plate with nitrocellulose membrane (alignment of strong color signals with separated plaques after reactivity between plaques and *Toxoplasma* high antibody titer pooled sera).

Table 3.3.1: Summary for the number of obtained plaques from 25 different plates and that were reactive with anti-*Toxoplasma* high antibody titer pooled sera.

Plates number	Number of reactive plaques	Total
1	25	
2	29	
3	19	
4	2	
5	30	
6	24	
7	5	
8	18	
9	3	
10	15	**329**
11	22	
12	10	
13	1	
14	4	
15	26	
16	9	
17	14	
18	2	
19	3	
20	16	
21	8	
22	22	
23	3	
24	12	
25	7	

3.4. Proving reactivity of isolated M13 phages by Dot-ELISA (Phage Dot-Blot Assay):

Before investing in M13 phage epitopes identification by DNA sequencing; the reactivity of selected M13 phage clones with anti-*Toxoplasma gondii* high antibody titer pooled sera were confirmed by Phage Dot-Blot Assay. The first of two NC membrane received (100μl, 50μl and10μl of different selected amplified phages) and the second NC membrane received (30μl and 10μl of different selected amplified phages). The spotted phages were in both membranes were reacted against diluted (1:500) anti-*Toxoplasma gondii* high antibody titer pooled sera, protein A–HRP was used in a dilution of 1:6000 as secondary antibody. The dot-ELISA results showed reactivity with most selected and amplified M13 phage. Dot-Blot Assay revealed and standardized for the confirmation of reactivity selected M13 phage clones with *Toxoplasma gondii* high antibody titer pooled sera. (figure 3.4.1 and 3.4.2).
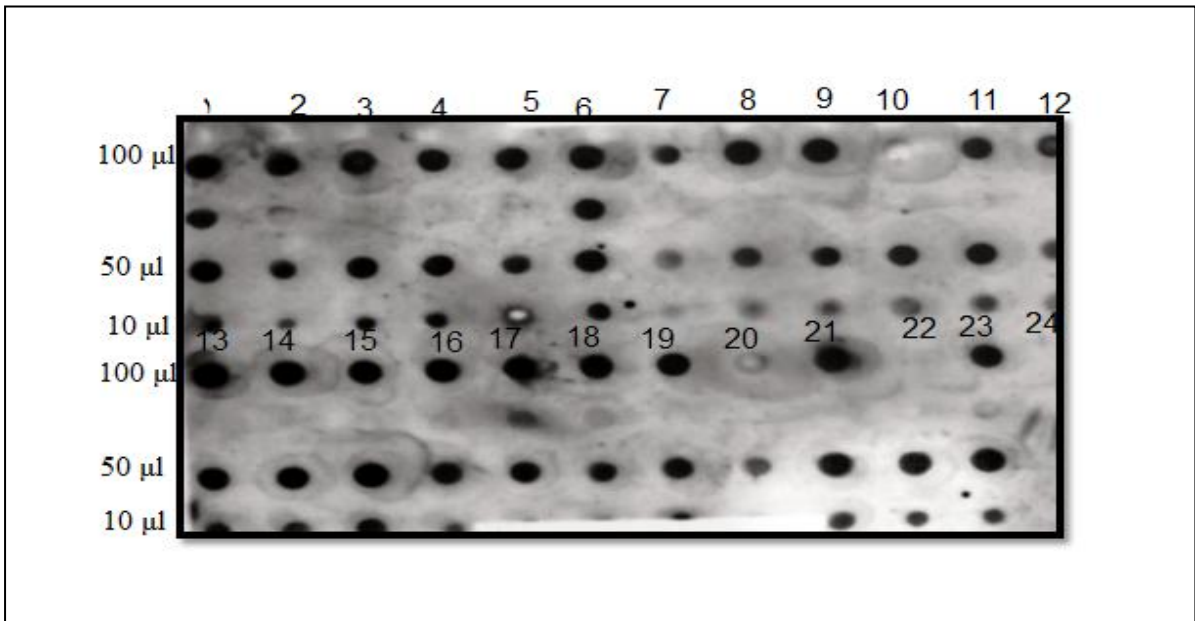
Figure 3.4.1: Screening of selected amplified M13 phage clones by dot-ELISA. Selected M13 phage clones suspended in TBS and blotted (100μl, 50μl and10μl) onto the nitrocellulose membrane. (1-23 are different selected phages reacted with *Toxoplasma gondii* high antibody titer pooled sera).
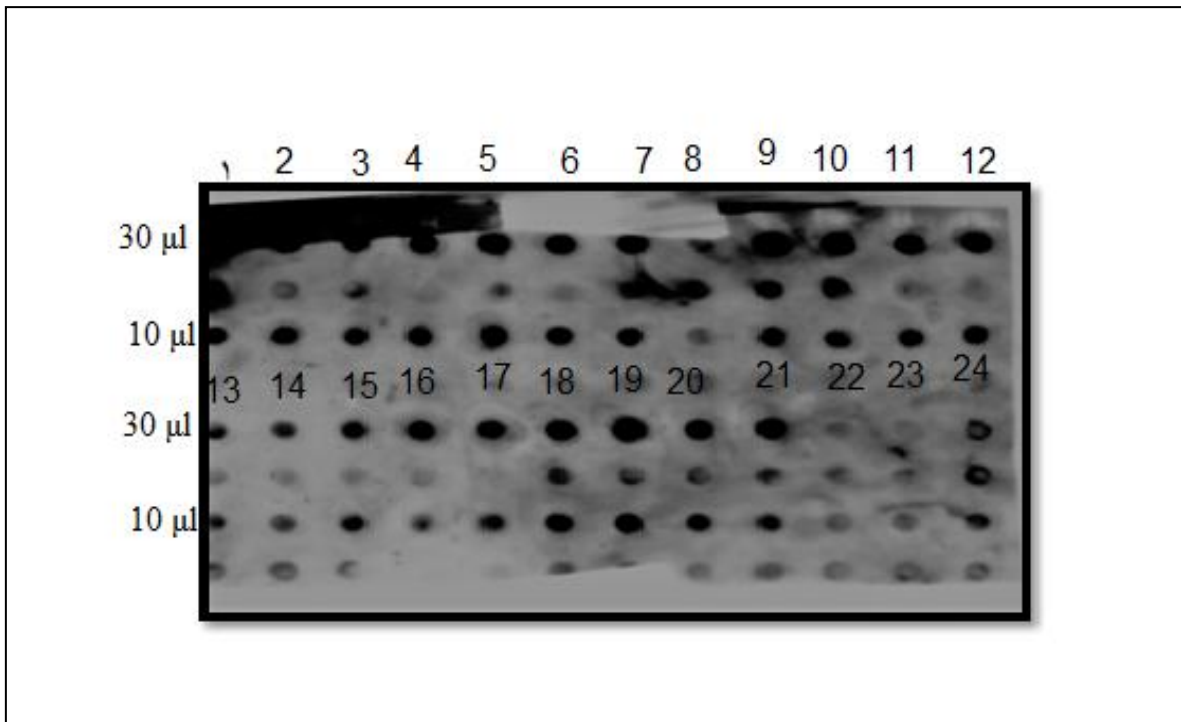
Figure 3.4.2: Screening of selected amplified M13 phage clones by dot-ELISA. Selected M13 phage clones suspended in TBS and blotted (30μl and 10μl) onto the nitrocellulose membrane**.** (1-23 are different selected phages reacted with *Toxoplasma gondii* high antibody titer pooled sera).

3.5. Phage / ELISA immunological reactivity assay:

Antibody titers of pooled sera reacted with amplified phages (reactive phages) were determined by ELISA assay using two plates with a difference in the used second antibody anti-human-IgG-HRP was used in the first plate and protein-A-HRP was used in the second plate. The results of this interaction showed high ELISA readings indicating high reactivity between isolate amplified phages and the pool sera. Based on this assay, the phage groups that produced the highest antibody titers were used to coat new ELISA plates. Five different groups were used to coat five different ELISA plates. These coated plates were examined for their reactivity with collected sera of *Toxoplasma gondii* infected individuals with other negative control sera. The results indicate that, the five selected phage groups used in this ELISA assay; showed consistency and high antibody titer in terms of their reactivity against the examined phages. (As shown in figure 3.5.1)

43
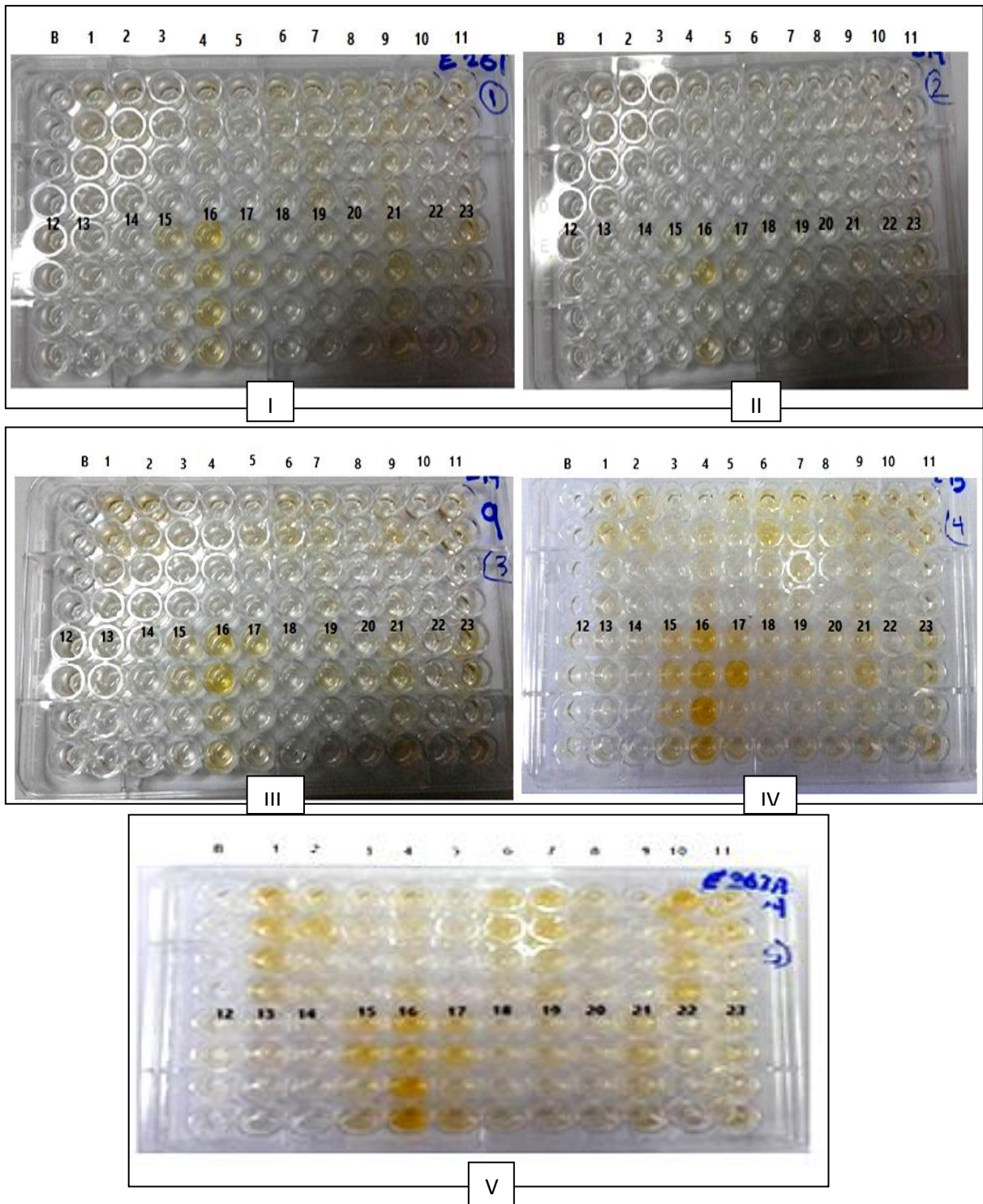
Figure 3.5.1: Phage ELISA Binding Assay for different sera of *Toxoplasma gondii* infected individuals reacted with different five amplified phages. I, II, III, IV and V: Represent ELISA immunoassay to different isolate concentrated phages and different sera of *Toxoplasma gondii* infected individuals. Protein A used as secondary antibody. B: is a Blank. No.5: is a positive control. No. 6: is pooled sera. No. 12&13: are Negative controls. No. 1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23: are different sera of *Toxoplasma gondii* infected individuals defined as high antibody titer.

3.6. M13 phage epitopes identification:

From a total of 138 selected M13 phage plaques that were chosen after three screening cycles with *Toxoplasma gondii* pooled sera, 24 different plaques were selected for sequence analysis and the Peptides coding sequences of the 24 different clones identified by high antibody titer *Toxoplasma gondii* pooled sera were obtained (sequences are shown in appendix A). The 24 plaques were selected based on their strong signals obtained after reactivity of the bound protein-A-HRP to the human antibodies from *Toxoplasma gondii* infected pooled sera that succeeded to identify the different M13 phage plaques. These plaques represent M13 phage epitopes that bind to antibodies against *Toxoplasma gondii*. Searching the GenBank; there was no DNA similarity or amino acid similarity between the obtained sequences and *Toxoplasma gondii* parasite DNA genomic sequences. But the sequences of the inserted clones which represent the selected M13 phages showed similarity to some related protozoan parasites such as *Plasmodium knowlesi* and *Plasmodium fragile.* (as shown in 3.6.3.1)

3.6.1. DNA amplification of the fused peptide/pIII M13 phage from the selected clones:

To identify selected plaques through DNA amplification of the fused peptide of pIII M13 phage, its known insertion site of the random peptides in the pIII M13 phage gene to form a sequence of random peptide library pIII fusion peptides. Based on this information of M13 phage library construction (New England Biolabs, Ipswich, MA, USA) and on the DNA sequence information of the pIII gene; a pair of primers were designed to enable a specific amplification of the fusion peptide (Figure 3.6.1). PCR primers were designed based on the information that is provided in the manufacturer company of the M13 phage library. At this stage; DNA was amplified using two primers that are flanking the peptide

region and amplifying a DNA segment about of 250bp (Table 3.6.1). Based on previous studies that showed it amplification of the inserted peptides by using Ph Rev2 and Ph Direct 2 primers that shown in table 3.6.1 and figure 3.6.1.

Ph Direct2 and Ph Rev2 primers were used in PCR amplification of the fused DNA, the Ph F and Ph Rev2 primers also were used in a combination in DNA amplification. But Ph Direct2 and Ph Rev2 primers revealed success in amplification of DNA segments to the selected clones more than Ph F and Ph Rev2 primers due to the Ph Direct2 and Ph Rev2 primers gave sharp bands compared to Ph F and Ph Rev2 primers. These PCR primer combinations (Ph Direct2 and Ph Rev2) were able to amplify about of 250bp DNA segment that include the inserted peptide and the flanking region from the pIII gene. PCR system was able to amplify the DNA segment from all the 45 selected clones. (As shown in figures 3.6.3 and 3.6.4)

Figure 3.6.1: DNA and amino acid sequence information of the pIII-peptide fusion in M13 phage. Demonstrating the location of two primers that were used in DNA segment amplification; Ph Direct2 : at 5'end and Ph Rev2: at 3'end. Direction of clone fusion: 5'end-3'end.

Table 3.6.1: Primers names and their DNA sequence; that were used in amplifying the pIII-peptide fusion DNA fragment from M13 phage.

Phage primers	DNA sequence	Size of the amplified DNA band
Ph F	ATT CAC CTC GAA AGC AAG CT	Using PhF and PhRev2 About (220bp)
Ph Direct2	TTA TTC GCA ATT CCT TTA GTG	Using PhDirect2 and PhRev2 (250bp)
Ph Rev2	CC CTC ATA GTT AGC GTA ACG	

```
5'-ggagcctttt ttttggagat tttcaacgtg aaaaaattat

   tattcgcaat tcctttagtg gtacctttct attctcactc

   T  [    Site of inserted peptide    ]

   gctacttcg catctgcatg ttcggttgcc gtcgaagggt

   ggaggttcgg ccgaaactgt tgaaagttgt ttagcaaaat

   cccatacaga aaattcattt actaacgtct ggaaagacga

   caaaacttta gatcgttacg ctaactatga ggga  -3'
```

Figure 3.6.2: DNA sequence of larger segment of the pIII M13 gene with the site of the inserted sequence. By the aid of this sequence of PIII M13 gene direct primer (Ph Direct2) near the 5'-end, and a reverse primer (Ph Rev2) near the 3'-end were designed to enable larger DNA fragment amplification.

3.6.2. DNA sequencing of the fused peptides pIII gene:

Twenty four clones were chosen from among 45 selected clones based on a third screening cycle with *Toxoplasma gondii* high antibody titer pooled sera for further analysis

and later for peptide identification. Namely these clones were (Ph1, Ph5, Ph7, Ph8, Ph10, Ph13, Ph14, Ph18, Ph19, Ph20 (old), Ph23 (old), Ph24 (old), Ph21, Ph22, Ph23, Ph24, Ph25, Ph26, Ph27, Ph28, Ph29, Ph30, Ph31and Ph32). Amplicons of indicated clones were purified and sent for DNA sequencing. After receiving the DNA sequences; it was shown that clones Ph28, Ph21, Ph25, Ph26 and Ph27 were not successfully sequenced. Analysis of DNA sequences was performed for the remaining 19 clones.
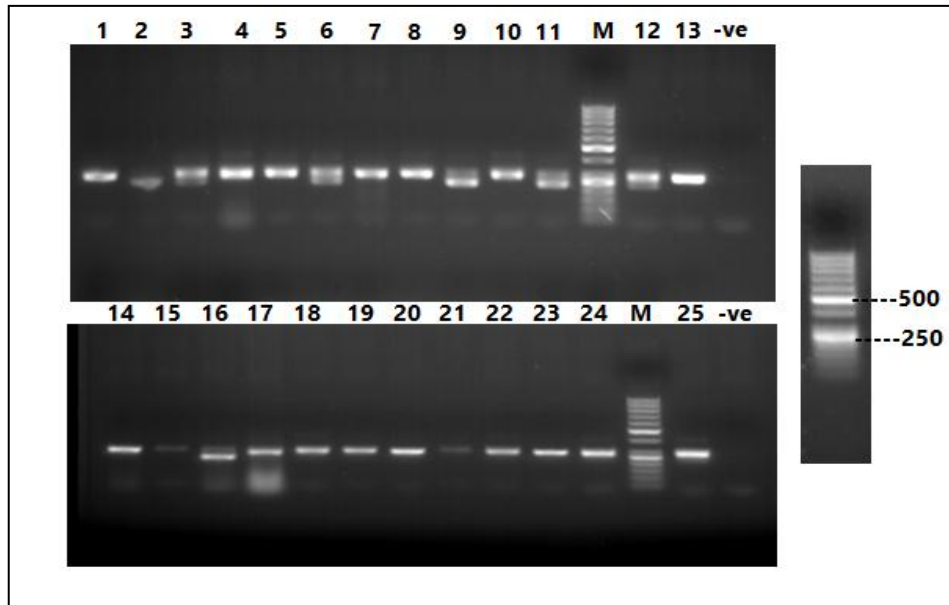
Figure 3.6.3: Agarose gel electrophoresis analysis of the amplified PCR products. Ph Direct2 and Ph Rev2 primers targeting the fused peptide in pIII M13 phage gene in the 25 different selected clones. Sample no.1 is ph1, no.5 is ph5, no.7 is ph7, no.8 is ph8, no.10 is ph10, no13 is ph13, no.14 is ph 14, no.18 is Ph18, no.19 is Ph19, no.20 is Ph20 old, no.23 is Ph23 old and no.24 is Ph24 old. –ve is a Negative control. M: Molecular weight marker.
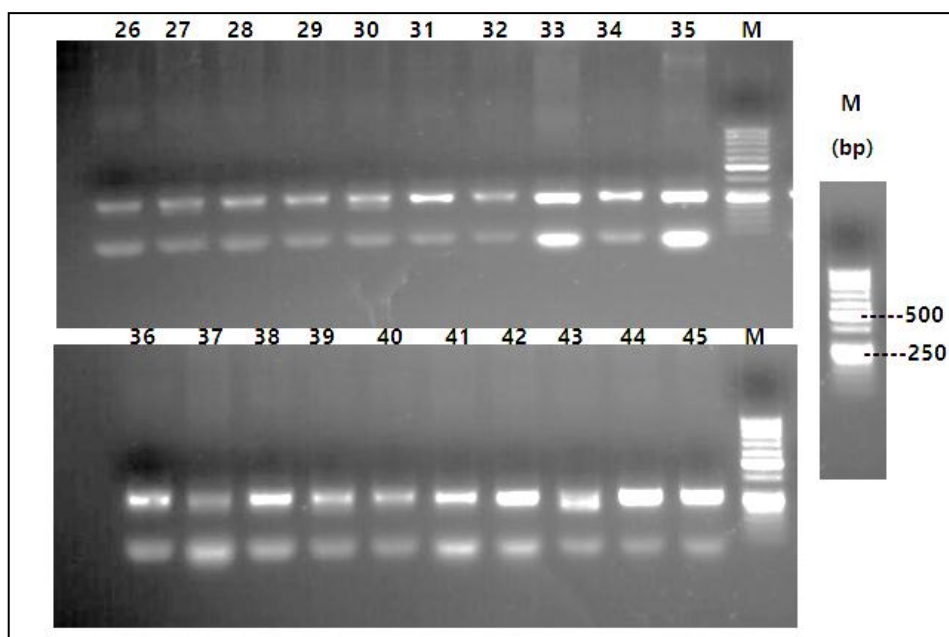
Figure 3.6.4: Agarose gel electrophoresis analysis of the amplified PCR products. Ph Direct2 and Ph Rev2 primers were targeting the fused peptide in pIII M13 phage gene in the 26-45 different selected clones. M: Molecular weight marker. Samples from no.26 to no.33 are ph21 to ph28. Samples no.36 and 37 are ph29 and ph30. Samples from no.41 and 42 are ph31 and ph32.

3.6.3. Bioinformatics and DNA sequence analysis:

Bioinformatics was used for the similarity searching to the 19 remaining putative peptide sequences through using BLAST online service provided through the PubMed /US National Institute of Health (http://www.ncbi.nlm.nih.gov/pubmed/).

Firstly, by the aid of BLAST program; the DNA sequence alignment of the 12 amino acid coding region including the flanking regions of the pIII gene of M13 phage was obtained. The sequences of 19 remaining putative peptides represent the selected M13 phages that showed cross-reactivity with anti-*Toxoplasma* pooled sera, 5'-end represented histidin and serine amino acids and those the 3'-end represented three glycine molecules. (As shown in figure 3.6.5)

From the 19 examined peptide DNA sequences, no clones showed similarity to *Toxoplasma gondii* parasite DNA genomic sequences. But the sequences of the inserted clones which represent the selected M13 phages that showed cross-reactivity with anti-

Toxoplasma pooled sera; which the sequences represent the selected M13 phages related to protozoan parasites such as *Plasmodium knowlesi* and *Plasmodium fragile.* (as shown in section 3.6.3.1)

The DNA sequence similarity data analysis for peptides by BLAST analysis showed a 100% similarity with *Blastocystis hominis* and *Echinostoma caproni*. Also, and in another region of the peptide sequence that represents 96% similarity with *Spirometra erinaceieuropaei*. Similarity data analysis for peptides also showed 92% similarity with *Plasmodium fragile*, *Spirometra erinaceieuropaei, Enterobius vermicularis* and *Diphyllobothrium latum*. Some of these parasites are protozoan parasites and are closely related to *Toxoplasma* family (Sarcocystidae). (as shown in 3.6.3.1)

```
ph30        GGTACCTTTCTATTCTCACTCT CANATTCNG--CNGNC-NGAGCNGGATCGTCGGCTGGG TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph20(old)   GGTACCTTTCTATTCTCACTCT TTTAGTACG--CGTTA-TCAGAGTGGGGATTTGCCGGA TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph7         GGTACCTTTCTATTCTCACTCT GGTTGGCTG--ACTGA-GTCGCATGCTTATATGCCGGC TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph14        GGTACCTTTCTATTCTCACTCT TATTCGATG--AGTGA-GGAGTTGTAGAAGGATCCGTG GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph13        GGTACCTTTCTATTCTCACTCT ATTCAGTCG--CTTCCGTTTAATGCTTCTGTTCGGTNC TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph32        GGTACCTTTCTATTCTCACTCT TCGAAGGAT--CTTACGAATNATGCGCGT-TTTAGGAT TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph18        GGTACCTTTCTATTCTCACTCT CATCTTATT--CGTATGAGTGGTGCTACGTCGTCTTCC GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph23(old)   GGTACCTTTCTATTCTCACTCT AGTCTTAAT--TCTGT-TCATCATCAGACTCGGGGGCT GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph29        GGTACCTTTCTATTCTCACTCT CAGCCGCNG--CCGGT-TCATCTTAAGAATACGTTTCT GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph8         GGTACCTTTCTATTCTCACTCT GATTGGTCG--GTGATTACGTCGTACTGTTTTGAGGTG GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph19        GGTACCTTTCTATTCTCACTCT ATTAATGTG--AATCA-GCATTCTGAGACGGTTAATGA GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph10        GGTACCTTTCTATTCTCACTCT NNTTTTCCG--GCTCC-TTATNNTNNTATNCAGTAGGC GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph1         GGTACCTTTCTATTCTCACTCT CGTGATGTG--GATCG-TTTGTTGCTTCATCAGAAGAA GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph24(old    GGTACCTTTCTATTCTCACTCT GATTATCAT--GATCC-GAGTCTGCCTACGCTGCGGAA GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph5         GGTACCTTTCTATTCTCACTCT GGTCNTNNTGGGATGACNAGGANTCAGAAtGtATGcaG GGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph22        GGTACCTTTCTATTCTCACTCT GCTAAAAGG--TTNAGCATGCNNGNCNGCTNCAGGNGC NGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph23        GGTACCTTTCTATTCTCACTCT CACGAGG--NTAAGCATGGTNGGCAGCTTCAGTCGT   TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph24        GGTACCTTTCTATTCTCACTCT CACGAGG--NTAAGCATGGTNGGCAGCTTCAGTCGT   TGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
ph31        GGTACCTTTCTATTCTCACTCT ---GAGN--CTNAGCATGNTGGGCANTNTCAGNCGN   NGGTGGAGGTTCGGCCGAAACTGTTGAAAGTTGTTTA
            ********************** 	                              ****************************************
```

Figure 3.6.5: DNA sequence alignment of the 12 amino acid coding region including the flanking regions of the pIII gene of M13 phage.
The sequences represents the selected M13 phages that showed cross-reactivity with anti-*Toxoplasma* pooled sera, At 5-end represents histidin and serine amino acids and those at the 3'-end represents three glycine molecules.

3.6.3.1. The DNA sequance similarity data analysis for peptides of clones:

1- *Plasmodium knowlesi* strain H chromosome 12, complete genome
　　Sequence ID: AM910994.1　　Identities:27/30(90%)

```
Query  2        TTCAGTCGCTTCCGTTTAAT-GCTTCTGTT  30
                ||||| ||||||||||||| | |||||||||
Sbjct  2876597  TTCAGGCGCTTCCGTTTAGTCGCTTCTGTT  2876626
```

2- *Plasmodium fragile* hypothetical protein partial mRNA
　　Sequence ID: XM_012480995.1　　Identities: 24/26(92%)

```
Query  4     CTTAATTCTGTTCATCATCAGACTCG  29
             ||| |||||| |||||||||||||||
Sbjct  2965  CTTCATTCTGCTCATCATCAGACTCG  2940
```

3- *Chistosoma margrebowiei*
　　ID: LL879171.1　　Identities: 29/33(88%)

```
Query  2      ATTGGTCGGTGATTACGTCGTACTGTTTTGAGG  34
              |||||| ||||||| || |||||||||||||
Sbjct  16430  ATTGGTTGGTGATTT-GTGGTACTGTTTTGAGG  16461
```

4- *Spirometra erinaceieuropaei*
　　ID: LN295640.1　　　　Identities: 25/28(89%)

```
Query  8    CGGTGATTACGTCGTACTGTTTTGAGGT  35
            || ||||||| || |||||||||||||
Sbjct  992  CGCTGATTAAGTGGTACTGTTTTGAGGT  965
```

5- *Spirometra erinaceieuropaei*
　　LN070735.1　　Identities: 23/25(92%)

```
Query  11   TGATTACGTCGTACTGTTTTGAGGT  35
            |||||| || |||||||||||||
Sbjct  721  TGATTAAGTGGTACTGTTTTGAGGT  697
```

6- *Enterobius vermicularis*
　　ID: LM416346.1　　Identities 23/25(92%)

```
Query  6     GTCGGTGATTACGTCGTACTGTTTT  30
             ||||||||||| | ||||||||||
Sbjct  6900  GTCGGTGATTACATTGTACTGTTTT  6924
```

7- *Diphyllobothrium latum*
   ID: <u>LL596793.1</u>   Identities: 23/25(92%)

```
Query  2      ATTGGTCGGTGATTACGTCGTACTG   26
              ||||||||||||| ||||| |||||
Sbjct  3669   ATTGGTCGGTGATCACGTCTTACTG   3645
```

8- *Echinostoma caproni*
   ID: <u>LL233882.1</u>   Identities: 20/20(100%)

```
Query  13     ATTACGTCGTACTGTTTTGA   32
              ||||||||||||||||||||
Sbjct  19322  ATTACGTCGTACTGTTTTGA   19341
```

9- *Blastocystis hominis*
   <u>XM_013043335.1</u>   Identities: 20/20(100%)

```
Query  16   TTGTTGCTTCATCAGAAGAA   35
            ||||||||||||||||||||
Sbjct  158  TTGTTGCTTCATCAGAAGAA   177
```

10- *Spirometra erinaceieuropaei*
   Identities: 22/23(96%)

```
Query  3      TGATGTGGATCGTTTGTTGCTTC   25
              ||||||||||||||||||| ||||
Sbjct  4403   TGATGTGGATCGTTTGTTCCTTC   4425
```

11- *Echinostoma caproni*
   ID: <u>LL233138.1</u>   Identities: 25/28(89%)

```
Query  7       GTGGATCGTTTGTTGCTTCATCAGAAGA   34
               || ||||||  |||||||||||||||||
Sbjct  129654  GTTGATCGGGTGTTGCTTCATCAGAAGA   129681
```

Part 4: Discussion and Conclusion.

Discussion:

The present study was performed to develop a suitable *Toxoplasma* immunological test based on identification of immunoreactive peptides of M13 Bacteriophage and to provide a continuous source of *Toxoplasma* antigenic material in sufficient quantities to be used in an ELISA technique or to be bound on synthetic latex or sheep red blood cells in order to perform agglutination test. This study was also performed due to the public health concern about toxoplasmosis. The disease has been reported in almost all the world, that affect up to one third of the human population in the world including the Mediterranean regions and neighboring countries; Arabian Gulf countries, Turkish, Beirut, Iran and Jordan. (Khammari, Saghrouni et al. 2013; Jones, Wilson et al. 2001; Montoya, Bennett et al. 2000). Previous studies have identified the prevalence of *Toxoplasma gondii* in Palestine (Hebron and Gaza cities), the studies showed high IgG and IgM antibody titers among pregnant women. (Al-Hindi and Lubbad 2009; Nijem and Al-Amleh 2009). The seroprevalence of *Toxoplasma gondii* in goats, sheep, chicken and turkeys were determined in Jenin, Tulkarm, Beer Sheva, Tel-Aviv and Jerusalem. (Salant et al. 2016**;** Othman and Al-Zuheir 2014)

The study also performed because of some imported commercial IgM test kits for toxoplasmosis had problems with specificity, resulting in high rates of false-positive test results, and these kits have low specificity and the reported results are misinterpreted (Jones, Wilson et al. 2001; Montoya 2002)**.** False positive IgM antibodies results may obtain if individual have Rheumatoid factor (RF) and antinuclear antibodies. (Montoya 2002; Abolghasem et al. 2011)

Infection caused by *Toxoplasma* parasite in human is characterized by the appearance of anti-*Toxoplasma* antibodies in the sera of the patients. The disease can be diagnosed in human by serological tests that includes the detection of IgM and IgG antibodies, or directly by molecular methods. (Paquet et al. 2013; Goldstein, Montoya et al. (2008); Hill and Dubey

2002) In our study, MAT was used to determine the antibody titers of the different tested serum samples against *Toxoplasma gondii* crude antigen. The MAT test utilizes formalized tachyzoites and it detects only IgG antibodies as mercaptoethanol used in the test destroys IgM antibodies. The test has been reported to demonstrate high sensitivity and specificity when compared to other serological assays. (Shaapan et al. 2008) All positive sera that gave 1:20 antibody titer in MAT test were collected in one pool. The pooling strategy was done, since the pool sera provide sufficient amount of anti-*Toxoplasma gondii* antibodies to carry out phage display libraries analysis and western blot at this study.

The reactive antibody with *Toxoplasma* crude antigen extract is IgG, since the Anti-human IgG and protein A (anti-human-HRP) were used as secondary antibodies during immunoblotting. Performing western blot technique in this study was important to enable planning for the screening of the phage library and to be sure about the reactivity or not of the high antibody titer positive human pooled sera collected from *Toxoplasma gondii* infected pregnant women with the different phage displayed epitopes.

Phage display is considered as the powerful methodology that includes production and screening a large number of random peptides of a specific length (8-20 amino acids) that expressed on the surface of phage. (Devlin and Panganiban 1990) In which peptides and proteins displaying on the surface of filamentous bacteriophage are the powerful methodology for protein identification, according antibodies are react with peptides and proteins displaying on the surface of filamentous bacteriophage. (Morozova and Tikunova 2009) Smith demonstrated that peptides on the surface of M13 bacteriophage can be expressed and affinity enrichment method termed "Biopanning" was developed. (Parmley and Smith 1988)

Theoretically, phage titration approach aims to enrich the plate with many plaques of the same type, and it is recommended to grow the phages in a titer that gives a lower number

of plaques per plate in order to enable easier isolation of independent plaques without any overlapping between them.

Blue-white screening allowing rapid detection of the plaques on the plate. A lawn of white-blue plaques on LB\IPTB\Xgal plate were obtained; blue plaques indicated that the amplified phages (viable phages) which the blue plaques resulted due to M13 bacteriophage carries lacZ gene, so phage plaques appear blue when plated on media containing IPTG and Xgal and blue plaques were picked rather than white plaques since the white plaques indicated that the sever contamination with the environmental M13-like phage during phage panning and amplification. (Messing Gronenborn et al. 1977)

After receiving the formations about DNA sequences; it was shown that the clone Ph28 was not successfully sequenced may due to mixed PCR amplification products. Ph21, Ph25, Ph26 and Ph27 also showed unidentified nucleotides through these sequences; these results obtained may due to overlapping between more than one picked reactive plaques or clones in these PCR amplified products.

The results of DNA sequencing showed no similarity of the obtained M13 phage epitopes to already known *Toxoplasma* proteins or sequences in the Gene Bank data base. This finding exclude the other obtained reactive peptides (sequenced or not) due to these peptides were bound to antibodies from *Toxoplasma gondii* infected pool sera. Epitopes considered as a small sequence of amino acids about 5-6 and these amino acids and have ability to form folding patterns also there are two types of epitopes; namely sequential epitope that based on sequence and conformational epitope that based on conformation. (Flanagan 2011; Huang and Honda 2006) In general most of selected and sequenced peptides had no linear sequence identities with any currently known proteins or DNA sequences and no complete consensus linear motif in common. This fact may eliminate the role of these peptides as sequential epitopes but never as conformational epitopes or as mimitopes that

mimic different antigenic proteins of similar amino acid composition, or carbohydrates that have similar conformational structure; which different amino acids can give the same of conformation of epitopes they can identify the antibodies. (Editorial 2001)

The sequences of the inserted clones which represent the selected M13 phages that showed cross-reactivity with anti-*Toxoplasma* pooled sera; which the sequences represent the selected M13 phages related to and identified as protozoan parasites such as *Plasmodium knowlesi* and *Plasmodium fragile*. The selected clones had also a cross reactivity with *Spirometra erinaceieuropaei*. Based on a previous study that was conducted to identify the early diagnostic antigens from *Spirometra erinaceieuropaei* using immunoproteomics showed that the *Spirometra erinaceieuropaei* might have similarty to *Toxoplasma gondii*, (Dan Hu, Xiao et al. 2014) also at this study, suggesting that *Toxoplasma gondii* might had similar gene sequences and common antigen with *Spirometra erinaceieuropaei*. Thus, the immune sera from pregnant women had cross immune reaction with *Spirometra* proteins.

Conclusion:

In conclusion, the epitopes of *Toxoplasma gondii* was recognized by immune sera of the pregnant women. The identified M13 phage epitopes has the potential for developing a suitable *Toxoplasma* immunological test based on identification of immunoreactive peptides of M13 bacteriophage by dot- ELISA and ELISA. The newly identified peptides can overcome the high antibody titers seen among negative controls examined by ELISA immunoassay. This study also provided a provision supply of *Toxoplasma* antigenic material to be used in an ELISA technique or to be bound on synthetic latex or sheep red blood cells in order to perform agglutination test.

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Appendix A

Peptides coding sequences of the 24 different clones identified by high antibody titer _Toxoplasma gondii_ pooled sera

>ph1

NNTTTGTCTCCTTCaCgAcGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAAC
AACTTTCAACAGTTTCGGCCGAACCTCCACCCTTCTTCTGATGAAGCAACAAACG
ATCCACATCACGAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAA
TAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAAT
TGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATAN

>ph5

TTTGTcgTCTTCaCgAGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAAC
TTTCAACAGTTTCGGCCGAACCTCCACCCCtgCATaCaTTCTGANTCCTNGTCATCC
CANNANGACCAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAAT
AATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATT
GTATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph7

ATTTGTcgTCtTTCaCGACGTTAGTAAATGAATTTTACTGTATGGGCATTTTGCTAA
ACAACTTTCAACAGTTTCGGCCGAACCTCCACCAGCCGGCATATAAGCATGCGAC
TCAGTCAGCCAACCAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAAT
AATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGANCNTTTA
NTTGNATCGGNTNATCNGNNTGNNTTCAAGGTGAATCNTATANCCGGNGTGNAN

>ph8

TTTGTcgTCTTCaCAcGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAAC
TTTCAACAGTTTCGGCCGAACCTCCACCCCACCTCAAAACAGTACGACGTAATCA
CCGACCAATCAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAATA
ATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTT
ATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph10

TTTGTCgTCTTCaCgACGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAA
CTTTCAACAGTTTCGGCCGAACCTCCACCCGCCTACTGNATANNANNATAAGGA
GCCGGAAAANNAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAA
TAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAAT
TGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph13

TTTGTCgTCTTCaCgACGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAA
CTTTCAACAGTTTCGGCCGAACCTCCACCAGNACCGAACAGAAGCATTAAACGG
AAGCGACTGAATAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATA
ATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAA
TTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph14

TTTGTcgTCTTCaCgAcGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAA
CTTTCAACAGTTTCGGCCGAACCTCCACCCCACGGATCCTTCTACAACTCCTCACT
CATCGAATAAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAATAA
TTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGT
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>ph18

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CGAATAAGATGAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAAT
AATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATT
GTATCGGTTTATCAGCTTGCTTTCGAGGTGAATANA

>ph19

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CAACTTTCAACAGTTTCGGCCGAACCTCCACCCTCATTAACCGTCTCAGAATGCT
GATTCACATTAATAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATA

ATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAA
TTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph20(old)

TTTGTcgTCtTTCaCAcGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAAC
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CGTACTAAAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAATAA
TTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATTGT
ATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph23(old)

TTTGTcgTCTTTCaCgACGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACA
ACTTTCAACAGTTTCGGCCGAACCTCCACCCAGCCCCCGAGTCTGATGATGAACA
GAATTAAGACTAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAAT
AATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATT
GTATCGGTTTATCAGCTTGCTTTCGAGGTGAATA

>ph24(old)

TTTGTCgTCtTTCaCgAGTTAGTAAATGAATTTTCTGTATGGGATTTTGCTAAACAA
CTTTCAACAGTTTCGGCCGAACCTCCACCCTTCCGCAGCGTAGGCAGACTCGGAT
CATGATAATCAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAATA
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>ph21

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GTCATGNGGGGGTGTGCTTNGNGTCTGTGTNGNCTCTGCNACGAATTCATGNNNT
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>ph22

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CNNGCATGCTNAACCTTTTAGCTGNNANNAGANCGGNACCACNAAGGNNNNTGC
AGGANCCATTTTTTGCACNNNNNAAANCTCNTGCNAAAGGCGTGAATAAGGAGC
CTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATANNNNNNNNNNNN

>ph23

GANTNTTGTACNCCTTNGCNTAGTTGNTAAATGAANTNNTANAGTATGGGNNNN
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TGGATGACGAGGANGCNTAGNCNTCTGNAGAGACGTNTAGAAAGGGNNCATGG
ATGGGNNTTTNNGAGNNNNAANNGGTGTCTGNGGGTTANTTTCNNNGNTGANGG
GGGGGGNTGNGANCNNGCNTGCGNANNNTNNNGATTAGCACTGGCTTNCNACG
GGGNNNNGAANGNGACTNNNCNTGNGAANTNNANANCACGCNNGNTCNGN

>ph24

ANACGNTTTGTACTCCTTNCCTACGTTGNTAAATGAATTTTACTGTATGGGCATTT
TGCTAAACAACTTTCAACAGTTTCGGCCGAACCTCCACCNANNAACGACTGAAG
CTGCCNACCATGCTTANCCTCGTGAGTGAGAATAGAAAGGTACCACTAANGGAA
TTGCGANTANTAATTTTTNCACGTNGAAAATCTCCANAAAAAAGGCTCCAAAAG
GAGCCTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATAANCNNGCNT

>ph25

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CTTTCAACAGTTTCGGCCGAGCNNCCACCAAAAGGCNCCNGNANCNGANTTGCA
GAGCNAAAANTTTTNCNGGTTGAANATCGGCACCAAAAAGGCNCCTGNNGGAGC
CATTNATTGNANNGGNTTANCNGCNTGCTTNNGNGGTGAATAGGACCCNTNAAT
TGNATCGGTTTATNAGCCNNGCNTTCCNAGGNTGAATAAANTAGGCNNATCNCN
ANGCCTNNNCGTCTTCANCTACNNGTGTNNNTTTANAACNGAGNCCNGCANATA

>ph26

GTGNNNCGCGNTCNNGTNANCGNTNNCTNTTNTNCTNTATNNCCNNTGGGNNGG
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CCCCCCCTCGATNNNNNNNCTTTCTATTTNTTNTNNTGCNCCATGCNGNNTCAGC
NCTGTNGTGCNTNTNTANGANGTGGTTANCGTGANGNTTACTNNANGNAATGGC

>ph27

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NANNATGCAGTNNCCGCTNGAGTGACGANTAGAANGGTNACCACATAATGGAAT
TGCNGGATNNATANTNTNTGCANGNTGANAATCTCCNGANAAAAGGCTNNANNN
GGAANNCTTTNATTGGTATCCGGTTTATCNNCTCGCTTTCTNCNTGNANANCNNC
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>ph29

AGACNTNNTGTANTCCTTNCCTAGTTNGTAAATGAATTTTACTGTATGGGATTTT
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GATGAACCGGCNGCGGCTGAGAGTGAGAATAGAAAGGTACCACTAAAGGAATT
GCGAATAATAATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGA
GCCTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATANNNCCAANCAN

>ph30

AAACGCTNTGTNNTCTTGCCTTACGTGGNNAAATGAATTTTANTGTATGGGCANT
TTGCTAAACAACTTTCAACAGTTTCGGCCGAACCTCCACCACCCAGCCGACGATC
CNGCTCNGNCNGCNGAATNTGAGAGTGAGAATAGAANGGTACCACTAAAGGAA
TTGCGNATAATANTTTTTTCACGTTGAAAATCTCCAGAAAAAAGGCTCCANAAGG
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GNGGTNNCNCANTNNTGGTANNGCNGNAGTANNANAANNTNGNCTNG

>ph31

NANACNATNTGTNNTCTTNCCTACGTTGGNAAATGAATTTTACTGTATGGGCATT
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ANTGCCCANCATGCTNAGNCTCAGAGTGAGAATAGAAAGGTACCACTAANGGAA
TTGCGAATAATAATTTTTNCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAG
GAGCCTTTAATTGTATCGGTTTATCAGCTTGCTTTCGAGGTGAATANNCNAGTNN
GCCTNNCTNTCGGNTTNTTGNTTNCANCTCTGCNNTTTTTTTNCGCCNNTGNNTCTN

CCNNTNCTTGNNGGCCGNANNCTNNCGNNCCGCNNNCTCCNCCNNTAGTTGNG
ANTGNGCNCNNTTAGNTT

>ph32

CNTTTGTACTCTNCCTAGTTNGTAAATGAATTTTCTGTATGGGANTTTGCTAAACA
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AGATCCTTCGAAGAGTGAGAATAGAAAGGTACCACTAAAGGAATTGCGAATAAT
AATTTTTTCACGTTGAAAATCTCCAAAAAAAAGGCTCCAAAAGGAGCCTTTAATT
GTATCGGTTTATCAGCTTGCTTTCGAGGTGAATANNNT

التعرف على المُعَلَمات السطحية لطفيل التوكسوبلازما من خلال استخدام امصال المصابين ذو التركيز العالي بالمضادات المناسبة للتشخيص المناعي.

إعداد: دينا نبيل خليل زيغان

المشرف الرئيس: د. إبراهيم عباسي

المشرف المساعد: د. رسمي أبو حلو

الملخص

يعتبر التوكسوبلازما جوندي (*Toxoplasma gondii*) طفيل احادي الخلية من عائلة المقوسات (Sarcocystidae)؛ حيث تتسلسل دورة حياته بين القطط والتربة والإنسان والحيوانات؛ فيستخدم القطط كعائل اساسي بينما يستخدم الانسان والحيوانات ذوات الدم الحار كعائل وسيط وهو المسبب لمرض داء المقوسات "داء القطط". ويؤثر هذا الطفيل على حوالي 30% من البشر الأمر الناتج عن توطنه في كثير من بلدان العالم مثل اوروبا والامريكتين ومناطق البحر الأبيض المتوسط .

تم إجراء هذا البحث للتعرف على المعلمات السطحية للتوكسوبلازما باستخدام تقنية العاثيات (Phages) بإستخدام النوع الخيطي (M13) من أجل الكشف والتعرف على الببتيدات (Peptides) التي تشبه بعض أنواع المعلمات السطحية لطفيل التوكسوبلازما من خلال ما يسمى بالبلاكس (Plaques) أو المناطق الناتجة من العاثيات والمكونة من 12 حمض أميني في ما يعرف بمكتبة العاثيات. ولإتمام هذه الدراسة تم جمع الأمصال (Sera) من النساء الحوامل المصابات، وتم تحديد العينات التي لها تركيز عالي من الأجسام المضادة من خلال إجراء فحص (MAT) ومن ثم تم تجميع العينات ذات التركيز الأعلى وعمل مجموعات (Pools). وقد تم بمساعدة هذه المجموعات (Pools) إجراء ثلاث دورات مسح لهذه العينات باستخدام العثيات (phages) المكونة من 12 حمض اميني وتم الحصول على 329 بلاكس متفاعلة مع هذه المجمعات في دورة المسح الثالثة ومن هذه البلاكس التي نتجت من دورة المسح الثالثة 138 بلاكس تم انتقاؤها وتم اختيار 45 بلاكس لمزيد من التحليل. ولقد تم عزل الببتيدات التفاعلية البلاكس التي اعطت تفاعلاً ايجابياً مع و pools وتكثيرها وإنتاجها في بلازميد مؤتلف واستخدامها في فحص الاليزا النقطية Dot-ELISA وأيضا في ELISA للكشف ان البلاكس (45) التي تم انتقاؤها بعد تفاعلها الايجابي مع ال Pools تشبه بعض انواع المعلمات

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السطحية لطفيل التوكسوبلازما وبالفعل من هذه الفحوصات تم الحصول على نتائج ايجابية تبين انه بالفعل يوجد بعض المعلمات من phage التي تشبه المعلمات السطحية لطفيل التوكسوبلازما.

وللتعرف على الشيفرة المكونة لهذه الببتيدات المكونة من 12حمض اميني تم استخدام الجين 3 من phage M13 والعمل على تكثيره باستخدام البارئات primers والتي تم تصميمها اعتمادا على الاطراف المحيطة لجين 3 ومن ثم تطبيق فحص PCR ومن ثم تم تحديد الترتيب القواعدي ل clones19 وبعد ذلك تم تحديد التشابه لهذا الترتيب من خلال البحث في بنك الموروثات باستخدام برنامج BLAST ومن هذا الترتيب القواعدي تبين ان العثيات المحددة M13 وجدت تفاعلية متصالبة Cross-reactivity مع المجمعات pools التي تحتوي على الاجسام المضادة لطفيل التوكسوبلازما , أي انه لا يوجد أي من 19 clones يشبه التوكسوبلازما تماما فهنا نحتاج للتفتيش عن سلسلة الحموض الامينية لهذه ال clones من خلال برنامج BLAST حيث تبين ان هذه ال clonesتشبه غيرها من الطفيليات مثل Plasmodium knowlesi و Spirometra . erinaceieuropaei لكن فحوصات Dot-ELISA و ELISA test اعطت نتائج جيدة لهذه ال Clones ومن هنا نستطيع استخدام هذه ال clones لدراسات اخرى. وهذه الدراسة ايضا وفرت كميات كبيرة من المعلمات المميزة للتوكسوبلازما والتي من الممكن استخدامها في المستقبل لعمل فحص ELISA او الصاقها على اللاتكس المصنعة او على كريات الدم الحمراء للأغنام واستخدامها لإجراء فحص agglutination test لعينات من البلد.