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# THE ROLE OF GRA8 ANTIGEN-DERIVED SYNTHETIC PEPTIDES IN SEROTYPING OF *TOXOPLASMA GONDII*

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# THE ROLE OF GRA8 ANTIGEN-DERIVED SYNTHETIC PEPTIDES IN SEROTYPING OF *TOXOPLASMA GONDII*

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

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January 2017

**ABSTRACT**

*Toxoplasma gondii* is an obligate intracellular parasite, capable of infecting humans and other warm-blooded animals. It is typically associated with the adequately named infection, toxoplasmosis.

The aim of the present work was to develop serotyping by selecting different synthetic GRA8 C-terminal polymorphic peptides with 64 human serum samples (of known strain types) to determine the peptides' ability to differentiate strain types of *T. gondii* (I, II, III and atypical).

Serotyping is a typing method consisting of an immunoenzymatic assay (enzyme-linked immunosorbent assay [ELISA]) using synthetic polymorphic peptides derived from *Toxoplasma gondii* antigens.

Sera samples were processed through indirect ELISA and the colour product formed measured by absorbance at 415 nm. Data obtained was processed in a Receiver Operating Characteristic (ROC) curve to determine the sensibility and specificity of each peptide.

Of the four GRA8II peptides used (217, 221, 246 and 258), only GRA8II 221 proved to have some ability to differentiate strains of type II from non-type II with an 85,8% probability of doing so. However, with such poor results for the remaining three peptides (all three with a probability of successfully distinguish between different strains types under 60%), GRA8 may not be an ideal peptide to be used for serotyping.

**Key words:** *Toxoplasma gondii*; GRA8; serotyping; polymorphic peptides

**RESUMO**

*Toxoplasma gondii* é um parasita intracelular obrigatório, capaz de infetar humanos e outros animais de sangue quente. *T. gondii* é tipicamente associado à infeção pela qual é responsável, designada por toxoplasmose.

O objetivo do presente trabalho foi testar novos péptidos polimórficos de GRA8 por serotipagem com 64 amostras de soro humano (com linhas clonais conhecidas) para determinar a capacidade dos péptidos de diferenciar linhas clonais de *T.gondii*.

A serotipagem é um método constituído por um ensaio imunoenzimático (ELISA) que utiliza péptidos polimórficos sintéticos derivados de antigénios de T. gondii.

As amostras de soro foram processadas através de ELISA indireta e o produto de cor formado foi avaliado medindo a sua absorbância a 415 nm. Os dados obtidos foram processados numa curva Receiver Operating Characteristic (ROC) para determinar a sensibilidade e especificidade de cada péptido.

Dos quatro péptidos de GRA8II usados (217, 221, 246 e 258), apenas o péptido GRA8II 211 demonstrou ter alguma capacidade para ser usado na distinção entre estirpes tipo II de estirpes não-tipo II, com uma probabilidade de 85,8% de o fazer com sucesso. No entanto, os resultados menos satisfatórios verificados para os restantes três péptidos (todos com uma probabilidade de distinguir com sucesso entre tipos de estirpes abaixo de 60%) sugerem que GRA8 talvez não seja o péptido mais indicado para ser usado em serotipagem.

**Palavras-chave:** *Toxoplasma gondii*; serotipagem; GRA8; péptidos polimórficos

**ACKNOWLEDGMENTS**

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# 1. INTRODUCTION

## 1.1 The Parasite: *Toxoplasma gondii*

*Toxoplasma gondii* is an obligate intracellular parasite, capable of infecting humans and other warm-blooded animals. It was first discovered in a Tunisian rodent in 1908 by Nicolle and Manceaux. It belongs to the kingdom Protista, subkingdom Protozoa, phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eimeriorina, family Toxoplasmatidae, genus *Toxoplasma* and species *gondii* (Dubey, 2010).

*T. gondii*'s life cycle is heteroxenous. Sexual reproduction is exclusive to members of the Felidae family, *T.gondii* definitive hosts (being the domestic cat the most common), while asexual replication can occur in both definitive and intermediate hosts. Virtually, all warm-blooded animals, including humans, can be intermediate hosts.

There are three infective stages of *T. gondii* - sporozoites (protected inside oocysts), tachyzoites, and bradyzoites (in tissue cysts) -, connected in a complex life cycle (Fig. 1). All three stages can infect both types of hosts (Dubey, 2010).

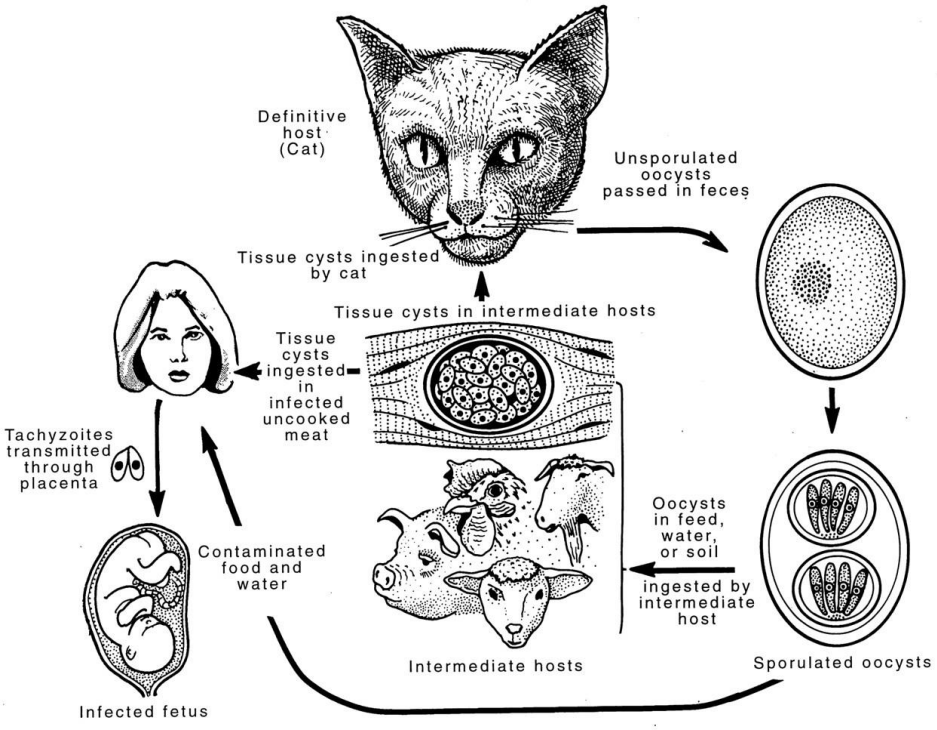


Figure 1: Life Cycle of *Toxoplasma gondii* (Dubey, 2010).

Sexual reproduction of *T.gondii* begins with the ingestion of any of the three infective stages (due to its high resistance, tissue cysts are the most common) by the definitive host. The parasite then invades the epithelial cells of the small intestine lining where it will undergo several rounds of division and differentiate into gametocytes.

The fusion of the gametocytes originates an oocyst that is shed into the environment with the host's faeces. After undergoing meiosis, oocyst produce sporozoites (Fig. 2) that, under the right conditions, may remain infective for up to a year in the environment (Halonen and Weiss, 2013).

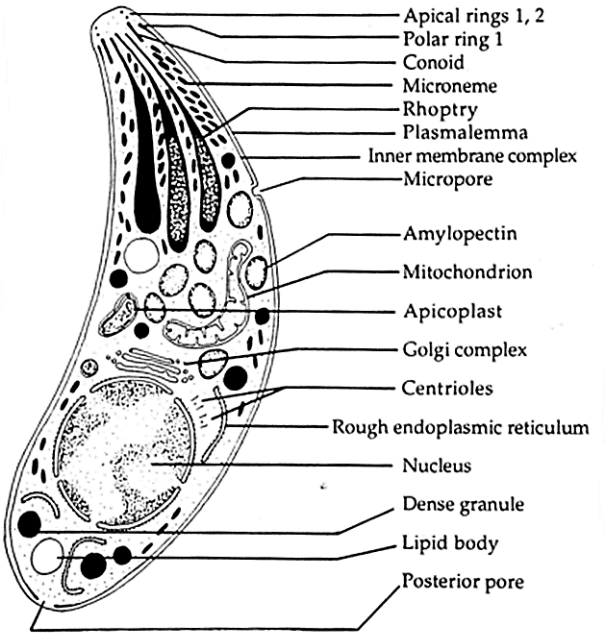


Figure 2: Schematization of a Sporozoite (Dubey, 2010).

The ingestion of sporozoites by any of the intermediate hosts marks the beginning of the asexual replication of *T.gondii*. After ingestion, the sporozoites go to infect the intestinal epithelium and differentiate into the tachyzoite stage.

Tachyzoites (tachos = speed in Greek) are crescent-shaped cells approximately 2 by 6 μm with a complex internal ultrastructure (Fig. 3). They are capable of rapidly multiplying inside any kind of nucleated cell by endodyogeny, after which the newly formed tachyzoites exit the cell and disseminate throughout the host (Halonen and Weiss, 2013). While they don't have visible means of locomotion (like cilia or flagella), tachyzoites can move by gliding; their motility is powered by an actin-myosin motor complex on the inner membrane (Blader *et al.*, 2015; Dubey,

2010). The activation of the host's immune system induces *T. gondii* to differentiate tachyzoites into bradyzoites and form tissue cysts.

Bradyzoites (brady = slow in Greek) represent the persistent stage of infection, as they may remain with the host through its life span. While tachyzoites aren't specific to a type of cell, bradyzoites are predominantly found in cerebral and muscle tissue (Robert-Gangneux and Dardé, 2012). Upon the loss of an immune response, bradyzoites can differentiate back into tachyzoites and resume their propagation through the host's cells.

Within the host cells, bradyzoites can be found inside tissue cysts. Whereas a bradyzoite in itself has similar dimensions to a tachyzoite, its cysts can reach up to be 100μm long and harbour thousands of bradyzoites (Dubey, 2010).

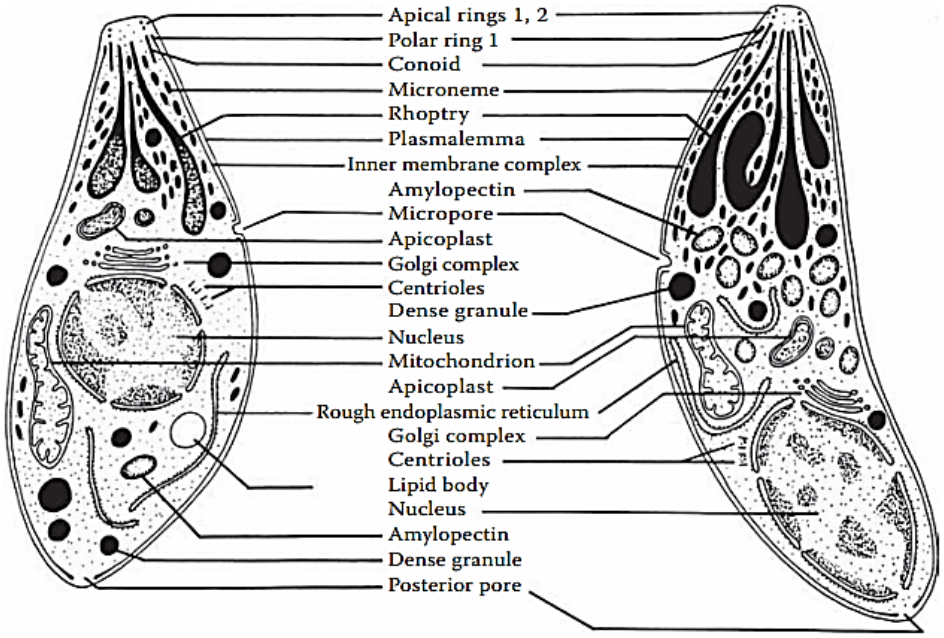


Figure 3: Schematic drawing of a Tachyzoite and a Bradyzoite, respectively (Dubey, 2010).

Despite having a heteroxenous life cycle, sexual reproduction of T.gondii it's not required for an effective transmission; therefore it can propagate asexually indefinitely (Dunn *et al.*, 2008).

## 1.2 Toxoplasmosis

*Toxoplasma gondii* is typically associated with the adequately named infection, toxoplasmosis. While the microorganism itself was first discovered in 1908, it was only successfully isolated from humans in 1939 by Wolf and colleagues, and only two years later, in 1941, was it proven by Sabin to be identical to the *T.gondii* that infected animals (reviewed in Dubey, 2008).

Toxoplasmosis can be acquired through different routes (Fig. 4). The most common routes are through ingestion of oocysts (sporozoites) from the environment and contact with cat faeces, ingestion of tissue cysts (bradyzoites) in undercooked meat, and by transplacental transmission of tachyzoites (Dubey, 2008). Other less common routes of transmission include accidental infection in laboratories while manipulating tachyzoites, blood (more specifically, transfusion of packed leukocytes) and bone marrow transfusions (both occurrences are theoretically possible if the donor was recently infected and is parasitemic at the time of sampling), as well as organ transplants since tachyzoites can infect all nucleated cells (Robert-Gangneux and Dardé, 2012). Due to such diversified routes of transmission, *T. gondii* can be spread from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive and between intermediate hosts (Tenter *et al.*, 2000).

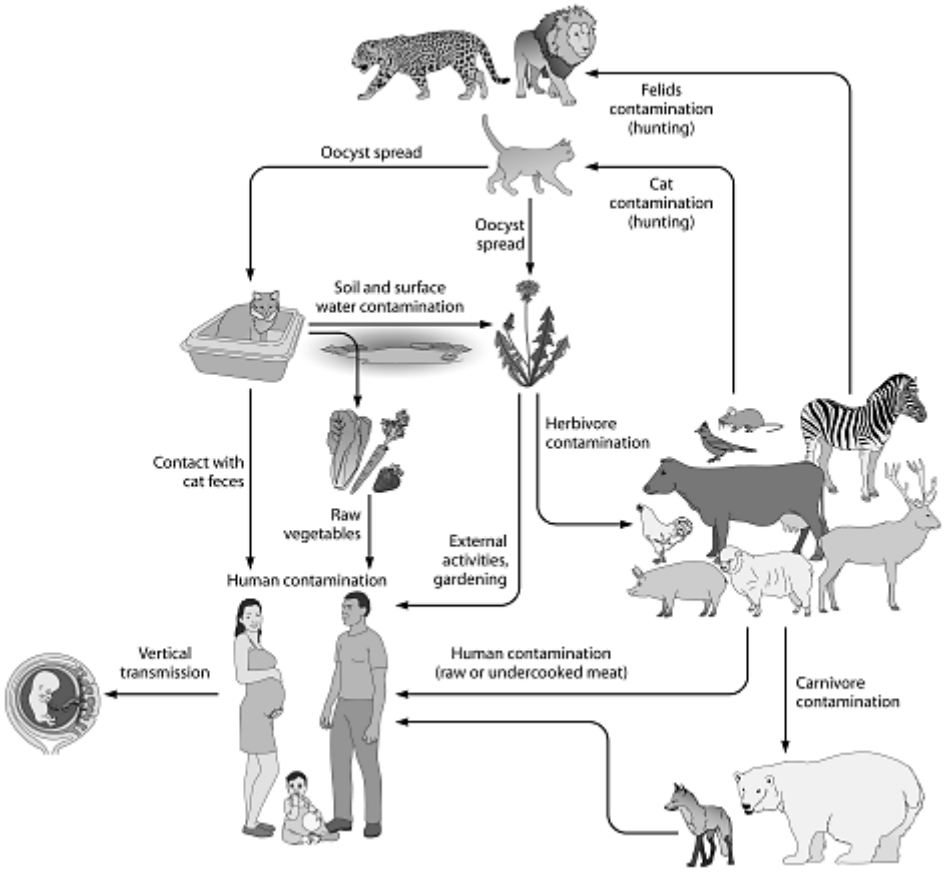


Figure 4: Sources of *T. gondii* infection in humans (Robert-Gangneux and Dardé, 2012).

In humans, *T. gondii* has to succeed in balancing the modification of its surrounding environment to support its survival and transmission and, simultaneously, avoid excessive tissue damage (either by the parasite, or unleashed by means of immune response) (Carruthers and Suzuki, 2007). Clinical manifestations of toxoplasmosis ensue from tissue destruction caused by the tachyzoites' fast-replicating stage, which could be lethal to the host (Blader *et al.*, 2015). In most *T. gondii*'s infections however, the parasite-host homeostasis is effectively maintained; for this reason, an infection by *T.gondii* usually remains asymptomatic in immunocompetent patients.

Those with clinical symptoms often experience lymphadenopathy in the head and neck region (axillary, inguinal, retroperitoneal and mesenteric lymph nodes may also occur); it may also be accompanied by fever, malaise, sore throat, rash and hepatosplenomegaly (Robert-Gangneux and Dardé, 2012; Halonen and Weiss, 2013). Although rarer, immunocompetent patients may display other more severe symptoms including encephalitis, sepsis, myocarditis, and hepatitis (Tenter *et al.*, 2000). Essentially, all organs can be affected. Toxoplasmosis has also been associated with neuropsychiatric disorders, which suggests that latent infection may have subtle neurological effects (Halonen and Weiss, 2013).

Because *T. gondii*'s bradyzoites may remain within the host through its life span - usually in a latent, subclinical infection -, there is a possibility of a spontaneous reactivation by means of differentiation of bradyzoites back to tachyzoites.

Whereas a functional immune system can prevent dissemination of tachyzoites, in immunocompromised patients (HIV/AIDS, transplants, and cancer patients) the immune system might be unable to respond accordingly. The chance of reactivation of latent infections is higher, symptoms deemed rare in immunocompetent patients are more common in immunocompromised individuals, and can be fatal if not recognized and promptly treated (Black and Boothroyd, 2000).

As mentioned before, toxoplasmosis can also be acquired through transplacental transmission of tachyzoites. This is called a congenital infection and is a consequence of primary infection of the mother during pregnancy. The earlier in pregnancy it occurs, the less likely are tachyzoites to colonize placental tissue and cross the placental barrier (Robert-Gangneux and Dardé, 2012; Sullivan and Jeffers, 2012). During the first trimester the risk of congenital infection is around 10% to 15%, while during the third trimester the risk increases exponentially to 60%–90% (Dubey, 2010).

The severity of the infection is not related to the degree of symptoms of the infected mother, but to the stage of pregnancy in which the infection occurred – the earlier in the pregnancy it occurs, the worst will the consequences be. The consequences of said infection on the foetus vary from subclinical (although clinical symptoms may develop later in life) to severe, leading to foetal loss or lesions involving, predominantly, the brain and eye.

### 1.3 Epidemiology: Prevalence of *T.gondii* in the World

*T. gondii* has a worldwide distribution which makes its associated infection one of the most common in humans and warm-blooded animals. It has been documented in virtually every species of mammal and even on several species of birds (Dubey, 2010). Prevalence of toxoplasmosis is higher in warm climates and in low altitude areas. This relates to the environmental conditions that favour sporulation and survival of oocysts.

Cultural habits and hygiene play an important role in the incidence of the infection, especially in terms of cooking and eating habits. It is estimated that about 50% to 80 % of the European population are infected (European Food Safety Authority (EFSA), 2016). For example, the high seroprevalence of toxoplasmosis in France is most likely related with eating raw or undercooked meat (especially pork and mutton). While in Asia - where most Asians cook their meat well - the seroprevalence in lower. In underdeveloped countries, the incidence of other parasites besides *T.gondii* also assures meat is cooked well prior consumption (Dubey, 2010), which lowers the incidence of the infection. The Centre for Disease Prevention and Control (CDC) estimates that, in the US, 22.5% of the population 12 years and older have been infected with Toxoplasma (CDC, 2015).

Although eating habits do play an important role in *T.gondii* transmission, it is unknown exactly what proportion of infection is caused by eating raw or undercooked meat contaminated with tissue cysts, and what is due to oocysts on unwashed hands or vegetables. It's also necessary to take into account the number and presence of the cats, climate, cultural and ethnic practices (Dubey, 2010).

## 1.4 Laboratory Diagnosis of Toxoplasmosis

The clinical symptoms of an infection by *Toxoplasma gondii* aren't specific enough to serve as a definite identification, so the diagnosis of toxoplasmosis relies on laboratory techniques. Toxoplasmosis can be diagnosed by biologic, serologic, and histological methods, or by a combination of some of these. Isolation of *T.gondii* can be attempted from different specimens like secretions, excretions, body fluids, and by biopsy of tissues (such as lymph nodes or muscle tissue) (Dubey, 2010).

Traditionally, detection of *T.gondii* relied on microscope examination. Light microscopy isn't sensitive enough to provide a reliable identification, but, samples can be enriched by filtration or centrifugation, and stained to help distinguish parasites from host cells. However, microscopic diagnosis is a time consuming method, may require considerable skills for the obtaining of reliable results and, therefore, isn't ideal for routine use (Liu *et al.*, 2015).

Isolating *T. gondii* by bioassay is generally considered as the gold standard for detection of *T. gondii* infection. Mice and cats are commonly used for bioassay of T. gondii (INF-gamma knockout mice due to high sensitivity to *T. gondii* infection; cats because they can be fed larger volumes of tissues, therefore increasing the sensitivity). Overall, a bioassay is expensive and time-consuming (usually requires 6 weeks). Hence why it isn't used for screening in large-scale.

Setting aside the previously mentioned diagnostic methods for being too time consuming (and therefore not ideal for routine use), and in combination with the lack of specific clinical symptoms, the diagnosis of a *T.gondii* infection usually falls on serological assays.

Over the years, several serologic tests have been developed - dye test (DT), modified agglutination test (MAT), enzyme-linked immunosorbent assays (ELISA), immunosorbent agglutination assay (ISAGA), indirect fluorescent antibody test (IFAT) and indirect haemagglutination assays (IHA) – to detect different antibody classes or antigens (Liu *et al.*, 2015).

In the present work, the serologic method used was ELISA.

ELISA is a biochemical technique used to detect the presence of an antibody or an antigen, in a sample, making use of an enzyme that reacts with a colourless substrate to produce a coloured product.

It begins with the absorption of a soluble antigen (in this case, a GRA8II peptide) onto a plastic surface, followed by the sera samples and the antigen-antibody reaction, enhanced by the addition of a secondary enzyme-linked antibody-antigen system. The reaction can be measured by quantification of the colour developed. In this method, the reacting enzyme is linked covalently to a specific antibody with the ability to recognize the target antigen. If present, the antibody–enzyme complex will bind to the antigen and, on addition of the substrate, the enzyme will catalyse the reaction generating a coloured product (Berg *et al.*, 2015).

## 1.5 *T.gondii* Antibodies and Antigens

Antibodies, also known as immunoglobulins (Ig), are proteins produced as a part of the immune response to identify and neutralise pathogens. Studying the kinetics of the different antibodies produced, especially IgM and IgG, can help identify the stages of a *T.gondii* infection (Saadatnia and Golkar, 2012).

IgM antibodies can be detected approximately 7 to 14 days after the onset of infection and persist for 1 to 6 months. IgM can remain detectable months or even years after acute infection, and for that reason can't be used as a reliable indicator of acute infection.

IgG antibodies are detectable days after the first positive IgM test, reaches a plateau within 2 to 3 months after the onset of infection which then decreases and persists with residual titres for life. The appearance and increase in IgG titre confirms the existence of an infection. The absence of IgM while IgG is still present indicates a past infection (over six months old), but it cannot accurately determine the timing of the infection (Gras *et al.*, 2004; Zhang *et al.*, 2016).

Antibodies can effectively identify and neutralise pathogens by recognising certain molecules on or within the pathogen cells responsible for the ongoing infection. These molecules are called antigens.

*T.gondii* has several classes of antigens that, through different functions, help the parasite successfully infect and colonise the host. Due to their role during the process of infection, these proteins are often used as targets for diagnosis of toxoplasmosis. Among them, there are five classes of antigens that are most commonly used for their immunodominance and for being present in both bradyzoite and tachyzoite stages: surface antigens (SAGs), matrix antigens (MAGs), micronemes (MICs), rhoptries (ROPs) and dense granules (GRAs) (Kotresha and Noordin, 2010).

SAGs establish the first contact of *T.gondii* with the host cells as they coat, like the name suggest, the surface of the parasite (Blader *et al.*, 2015). These molecules are also capable of inducing the host's immune response against the tachyzoite stage to, apparently, regulate the virulence of the infection (Jung, 2004).

MICs are involved in the recognition and adhesion of the parasite to the host's cells (Mercier, 2005; Kotresha and Noordin, 2010). They also play a role in the motility and egression (from the host cell, after replication) of the parasite (Blader *et al.*, 2015).

ROPs are discharged once MICs have successfully attached to the host cells. They are composed by two different substructures: rhoptry neck and rhoptry bulb. Rhoptry neck proteins assemble together with MICs to promote the formation of the moving junction - a tight apposition of the parasite and host plasma membranes visible during cell invasion that ensures formation of the parasitophorous vacuole membrane (Kotresha and Noordin, 2010).

GRAs are parasitic organelles involved in cell invasion and in the intracellular survival of the parasite (Sousa *et al.*, 2009). GRAs are released into the parasitophorous vacuole after invasion and are involved in its maturation, dictating the structure of the intravacuolar network of tubular membranes inside the parasitophorous vacuole where the parasite can multiply (Kotresha and Noordin, 2010).

Among all the GRA proteins of *T.gondii* (there are, at least, 40 identified GRA so far), GRA8 was selected for this experimental work because no previous serotyping studies on this antigen were found.

GRA8 is an intron-free gene with an open reading frame of 801 bp. The sequence of GRA8 is composed of 267 amino acids with 12 polymorphic positions, and contains an amino terminal signal peptide, three degenerate proline-rich repeats in the central region and a potential transmembrane domain near the carboxyl terminus (Carey *et al.*, 2000).

GRA8 is also an immunogenic protein, acting as a marker of acute infection. Since antibodies against GRA8 can be found during the acute phase of infection, IgG and IgM ELISAs with the GRA8 protein can be used to detect an infection of *T.gondii*, as well as discerning acute from chronic infections (Li *et al.*, 2000; Suzuki *et al.*, 2000).

## 1.6 Genotyping of *Toxoplasma gondii* isolates

Genotyping studies are based on the detection of polymorphic DNA markers and are independent of the immune response. However, strains of *T. gondii* are highly similar antigenically which makes it harder to distinguish the type of strain just through genotyping. The discovery of serological epitopes that are strain specific allows genotyping to be done based on serological profiles (Nowakowska *et al.*, 2006). Studies that help distinguish different types of strains are important to shed light into the biodiversity of the parasite and help establish a correlation between the genotype of *T.gondii* and the pathogenicity of the infection it causes in humans (Sousa *et al.*, 2009).

Genotyping methods are based on Polymerase Chain Reactions (PCRs) that can be divided in two groups. The first group consists of conventional, nest and real times PCRs and focuses on detecting *T.gondii* DNA; the second group consists of molecular methods including PCR-Restriction Fragment Length Polymorphism (RFLP), microsatellite analysis and multilocus sequence typing, which are mostly used for strain typing. The methods based on PCRs are usually simple, sensitive and reproducible, and can be used on all clinical samples. However, genotyping is not standardized yet and protocols, necessary equipment, DNA markers and target genes may vary (Ivović *et al.*, 2012).

## 1.7 Serotyping of *Toxoplasma gondii* isolates

The strain type is thought to play a role in the severity of an infection, and it's usually determined by genotyping. However, the principal limitation of genotyping methods is that it is hard to obtain infective isolates (with the exception of some severe cases or in pregnant women with a positive amniocentesis) (Morisset *et al.*, 2008). It requires isolation of enough parasite DNA, or actual organisms, and, generally, can only be acquired from patients presenting clinical manifestation of the infection (Kong *et al.*, 2003).

Serologic methods can get around this obstacle because they target antibody classes or antigens, not DNA. As it has been explained previously, *T.gondii* is capable of inducing a strong immune response where antibodies (usually IgG and IgM) can remain in high concentrations for months up to several years, or even during the host's whole life. The presence of said antibodies is also not dependent on whether the patient has symptoms of toxoplasmosis or not (Kong *et al.*, 2003).

With serotyping, specific polymorphic peptides derived from *T.gondii* antigens can be used to determine strain types in non-invasive and large-scale investigations with virtually an unlimited number of samples.

Several studies on this matter (such as Kong *et al.*, 2003 and Peyron *et al.*, 2006) have successfully been able to distinguish type II from non-type II infections (there is a the lack of differentiation between type I and type III due to high levels of homology) using synthetic peptides from other GRA proteins (like GRA5, GRA6 and GRA7) and other *T.gondii* antigens (Xiao *et al.*, 2009).

The main obstacle hindering serotyping from achieving all its potential lies in the fact that, for any given genetic locus, there are usually only two allelic types represented, and each archetypal line possesses a unique combination of those alleles (Kong et al., 2003). Which means that, for any given antigen, only two discriminatory peptides derived from polymorphic, immunodominant sites are required to determine which allele is carried by the infecting strain. The difficulty resides on finding these antigens and then synthetize the necessary polymorphic peptides.

## 1.8 *T.gondii* Clonal Lineages

*T.gondii* has three clonal archetypal lines: I, II and III. The origin of these three lineages seems to be the sexual cross between ancestral lineages. It was suggested by Boyle and colleagues (2006) that the origin lies on two separate crosses between ancestral versions of today's type II strain and two different strains named by the authors as α and β.

A cross between a type II ($II_1$) and a α-strain resulted in the creation of the type I strain. The type III strain resulted from a cross between another type II ($II_3$) strain and the β-strain (Fig. 5).

The fact that identical genotypes can be found in different countries and in different hosts, also supports the theory of today's strains being a product of a common clonal descent (Sousa *et al.*, 2008).

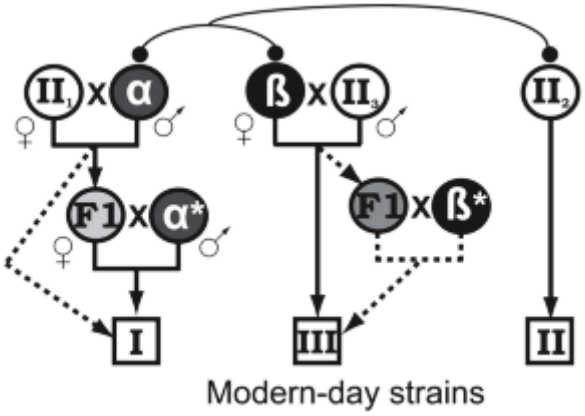


Figure 5: Schematization of the origin of the three *T. gondii* clonotypes (Boyle *et al.*, 2006)

Besides the three main strain types, several studies demonstrated the existence of strains that don't fit into any of the three types. These are non-archetypal strains and are commonly called atypical strains. Types I, II, and III lineages are predominant in Europe and North America. Non-archetypal strains with atypical genotypes are more frequent in other geographical areas, such as Africa and South America (Sousa *et al.*, 2008).

The aim of this work was to determine if the selected polymorphic GRA 8II peptides can be used to identify the strain type of the sera samples through serologic methods.

## 2. METHODOLOGY

### 2.1 *T.gondii* Sera Samples

The sera samples used came from three distinct geographical areas: Europe, Africa and Latin America (France (32 sera), Portugal (3 sera), Mexico (18 sera) and Africa (11 sera)).

Because the aim of this work was to determine if it was possible to conclude on the strain type trough serologic methods, all samples were previously genotyped and strain type was already known. Human serum samples had the following genotypes: (i) 12 cases associated with type I; (ii) 32 type II strains; (iii) 12 type III; (iv) 8 cases associated with atypical strains (See table 5-1 in the Attachments section).

20 sera samples were used as negative controls.

### 2.2 GRA8II Peptides

Four synthetic GRA8 C-terminal polymorphic peptides were used in this project. The peptides were named after the position in the coding sequence of the first amino acid of each peptide (Fig. 6 and Table 2.1-1).

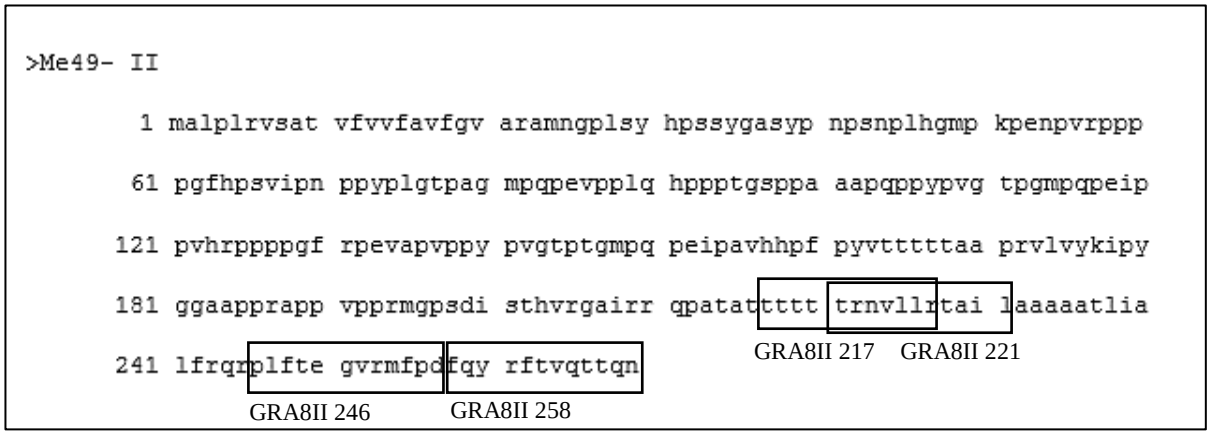


Figure 6: GRA8II Sequence; the sequences of the peptides used are marked with boxes.

Table 2.2-1: Sequences of the GRA8II peptides used; underlined type indicates known polymorphic sites of each peptide.

| Peptide | Loci | aa Position | Sequence |
|---|---|---|---|
| **GRA8II 217** | GRA8 | 217-227 | TTTTTRNVLLR |
| **GRA8II 221** | GRA8 | 221-231 | TRNVLLRTAIL |
| **GRA8II 246** | GRA8 | 246-257 | PLFTEGVRMFPD |
| **GRA8II 258** | GRA8 | 258-269 | FQYRFTVQTTQN |

## 2.3 ELISA

Several trial ELISAs were performed in the beginning of this experimental work to determine the best methodology. Different variations of the blocking agent, and several concentrations of Phosphate-buffered saline (PBS)-Tween solution, IgG alkaline phosphatase antibody, peptides and sample dilutions were tried. The best results were obtained with the methodology presented below.

Peptides were diluted in a carbonate/ bicarbonate buffer (0,05M; pH=9,6) to a concentration of 30 µg/ml. Nunc Immobilizer Amino plates (Thermo Scientific, Denmark) were coated with the diluted peptide and incubated in the fridge overnight at 4ºC. Wells were washed three times with PBS and blocked with a solution of 5% Bovine serum albumin (BSA) in PBS, followed by incubation in humid chamber at 37ºC for one hour. The plate was washed three times with PBS – 0,3% Tween 20.

The sera samples were diluted 1/50 in PBS – 3% BSA – 0,3% Tween 20, inoculated on the plate and incubated in humid chamber at 37ºC for two hours. Once completed the incubation period, wells were washed again three times with PBS – 0,3% Tween 20. Anti-Human IgG alkaline phosphatase antibody (Sigma-Aldrich, USA) was diluted 1/10000 in PBS – 0,3% Tween 20 and inoculated on the plate, followed by incubation in humid chamber at 37ºC for one hour. Well were washed again three times with PBS – 0,3% Tween 20 and developed with a solution of ρ-Nitrophenyl Phosphate (PNPP) in an Alkaline phosphatase buffer (NTMT) at 37°C for 10-15 min. Absorbance of the samples was measure at 415 nm using a plate reader (Bio-Rad, United States).

Blanks, consisting of PBS – 3% BSA – 0,3% Tween 20 + $dH_2O$, were used on the first and last well of each row of each module of the plate.

## 2.4 Statistical Analysis of Data Collected

Statistical analysis of the data collected was performed using GraphPad Prism version 6.1 for Windows.

Making use of several tools within the GraphPad Prism program, the Receiver Operating Characteristic (ROC) was performed, and optimal cut off values and sensitivity and specificity for each GRA8 II peptide were determined.

## 3. RESULTS AND DISCUSSION

The absorbance results of the ELISAs can be found on the Table 5-2, in the attachment section.

As mentioned in the Methodology section, statistical analysis was performed using GraphPad Prism version 6.1 for Windows.

The Receiver Operating Characteristic (ROC) curve was performed to assess the ability of each peptide to differentiate the different clonal lineages of *T.gondii*.

The ROC curve essentially evaluates the sensitivity and specificity of a test, or its true-positive rate versus false-positive rate. The higher the curve of a test, the greater is its discriminative ability. A test with low sensitivity but high specificity means that the positive results are reliable (low number of false positives), but the negatives aren't as reliable (high false positives). The inverse is verified if the test has high sensitivity but low specificity (low false negatives, but high false positives). An ideal test has both high sensitivity and high specificity.

The optimal cut off values can be determined with the help of the Youden Index (J), that here serve the purpose of helping define the maximum potential effectiveness of the peptide.

$$J = Sensitivity_x + Specificity_x - 1 \quad (3.1)$$

X represents sensitivity and specificity values for each point on the ROC curve.

The sensitivity and specificity values of each point are calculated by GraphPad Prism. By applying the equation (3.1) on those values, the maximum value of J can be determined; the values of sensitivity and specificity corresponding to said value, have a corresponding point on the ROC curve that is the cut-off point. The cut-off points, and respective sensitivity and specificity, of each peptide are presented on Table 3-1.

Table 3-1: Youden Index, sensitivity, specificity and cut-off values for each GRA8 II peptide.

| Peptide | **GRA8II 217** | **GRA8II 221** | **GRA8II 246** | **GRA8II 258** |
|---|---|---|---|---|
| Youden Index (%) | 113,38 | 145,88 | 104 | 116,5 |
| Sensitivity (%) | 59,38 | 71,88 | 50,00 | 62,50 |
| Specificity (%) | 55,00 | 75,00 | 60,00 | 55,00 |
| Cut-Off Value | 0,2865 | 0,3215 | 0,1870 | 0,3105 |

The area under the ROC curve (AUR), determined with the GraphPad Prism software, is, in this case, a reflection of how good each peptide is at distinguishing *T.gondii* type II from non-type II sera samples. The closer the ROC curve of the peptide is of the ROC curve when specificity + sensitivity =1 (which translates into an uninformative test, represented in the graphics of Fig.7 as a dotted line), the less accurate is the peptide.

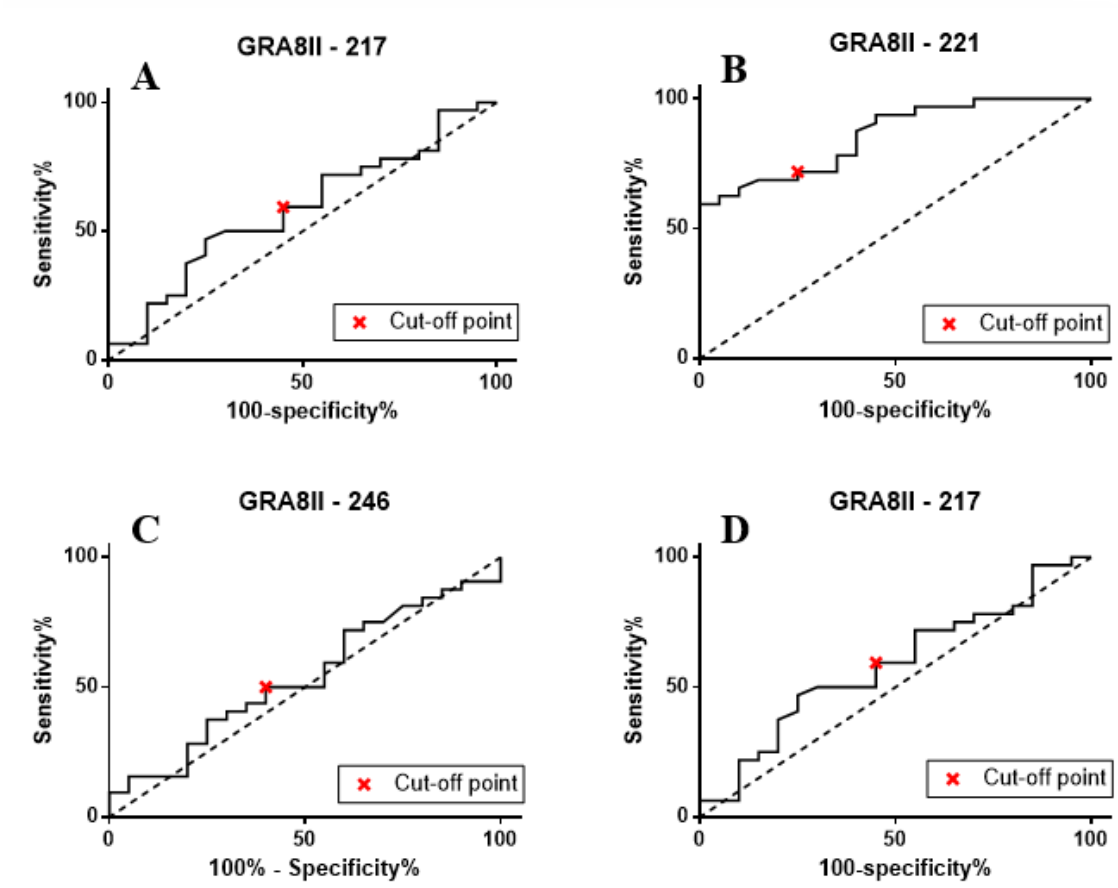


Figure 7: ROC curves for type II sera samples, for each of the four GRA8II peptides

The AUC for the GRA8II 217 peptide is 0,5828 which translates, roughly, into a 58,3% probability of the sera samples being of type II. Peptides GRA8II 246 and 258 present similar results to the GRA8II 217, with AUCs of 53,8% and 58.1%, respectively. Knowing that the genotype of each sample as already predetermined and the samples did belong to the type II genotype, adding to it the fact that the ROC curves formed by those peptides (Graphics A, C and D in Fig.7) stand too close to the [specificity + sensitivity =1] ROC curve, it demonstrates that the three peptides GRA8II 217, GRA8II 246 and GRA8II 258 cannot be used as a reliable tool to distinguish genotypes types.

GRA8II 221 has an AUC of 85,8%, and is the peptide whose ROC curve stands the furthest away from the [specificity + sensitivity =1] ROC curve (Graphic B in Fig. 7). Therefor it can be considered a good peptide (although not excellent) to be used to distinguish genotypes types.

The optical density (OD) of the ELISAs was measured at 415 nm for *T.gondii* positive sera of type I (I), type II (II), type III (III), atypical (At), and *T.gondii* negative sera (N), for each GRA8II peptide (Fig.8). OD values were calculated by subtracting the OD of the peptide control from the OD of each peptide.

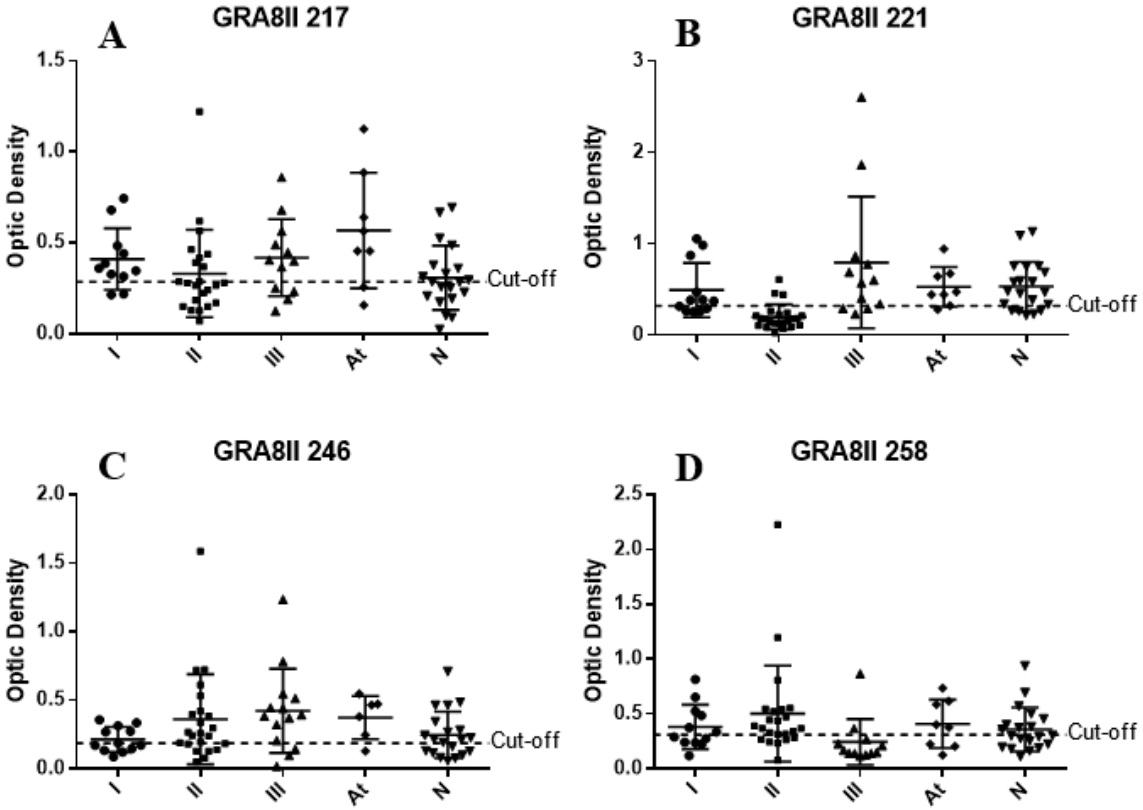


Figure 8: Distribution of optical densities in *T.gondii* positive sera of type I (I), type II (II), type III (III), atypical (At), and in T.gondii negative sera (N) for each of the four GRA8II peptides; the dotted line marks the cut-off for each peptide.

Originally, besides the four GRA8II peptides, there were eight more peptides planed (four GRA8I and four GRAIII). However, they were not synthetized in useful time and therefore could not be used in this experimental work. The decision to start with the type II peptides first was due only to the fact that type II *T.gondii* is more prevalent in Europe that any of the other two types.

Overall, taking into consideration the relatively low sensitivity and specificity values obtained for the GRA8II, it might not be the ideal peptide for the purpose of identifying types of genotypes.

## 4. CONCLUSIONS AND FUTURE WORK

Peptides to be used in serotyping must be polymorphic and immunogenic, which limits the peptides to specific regions of the protein. Although one of the four GRA8II peptides used – GRA8II 221 – fared relatively well in predicting type II sera samples, the apparent limited rate of polymorphisms in GRA8 peptide may not make this antigen the most suited for serotyping.

Future work on this matter may include the synthesis of the remaining eight GRA8 peptides initially intended to also be a part of this experimental work, to determine with certainty the ability of GRA8 to be used for serotyping.

## 5. ATTACHMENTS

Table 5-1: *T.gondii*-Positive Sera Samples Used

| SERUM | ORIGIN | STRAIN TYPE |
|---|---|---|
| TGp-II-01 | France | II |
| TGp-II-02 | France | II |
| TGp-II-03 | France | II |
| TGp-II-04 | France | II |
| TGp-II-05 | France | II |
| TGp-II-06 | France | II |
| TGp-II-07 | France | II |
| TGp-II-08 | France | II |
| TGp-II-09 | France | II |
| TGp-II-10 | France | II |
| TGp-II-11 | France | II |
| TGp-II-12 | France | II |
| TGp-II-13 | France | II |
| TGp-II-14 | France | II |
| TGp-II-15 | France | II |
| TGp-II-16 | France | II |
| TGp-II-17 | France | II |
| TGp-II-18 | France | II |
| TGp-II-19 | France | II |
| TGp-II-20 | France | II |
| TGp-II-21 | France | II |
| TGp-II-22 | France | II |
| TGp-II-23 | France | II |
| TGp-II-24 | France | II |
| TGp-II-25 | France | II |
| TGp-II-26 | France | II |
| TGp-II-27 | France | II |
| TGp-II-28 | France | II |
| TGp-II-29 | France | II |
| TGp-II-30 | France | II |
| TGp-II-31 | France | II |
| TGp-II-32 | France | II |

Table 5-1: T.gondii-Positive Sera Samples Used (cont.)

| SERUM | ORIGIN | STRAIN TYPE |
|---|---|---|
| TGp-I-01 | Africa | I |
| TGp-I-02 | Mexico | I |
| TGp-I-03 | Mexico | I |
| TGp-I-04 | Mexico | I |
| TGp-I-05 | Mexico | I |
| TGp-I-06 | Mexico | I |
| TGp-I-07 | Mexico | I |
| TGp-I-08 | Mexico | I |
| TGp-I-09 | Mexico | I |
| TGp-I-10 | Mexico | I |
| TGp-I-11 | Mexico | I |
| TGp-I-12 | Mexico | I |
| TGp-III-12 | Portugal | III |
| TGp-III-01 | Portugal | III |
| TGp-III-02 | Portugal | III |
| TGp-III-03 | Africa | III |
| TGp-III-04 | Africa | III |
| TGp-III-05 | Mexico | III |
| TGp-III-06 | Mexico | III |
| TGp-III-07 | Mexico | III |
| TGp-III-08 | Mexico | III |
| TGp-III-09 | Mexico | III |
| TGp-III-10 | Mexico | III |
| TGp-III-11 | Mexico | III |
| TGp-At-01 | Africa | Atypical |
| TGp-At-02 | Africa | Atypical |
| TGp-At-03 | Africa | Atypical |
| TGp-At-04 | Africa | Atypical |
| TGp-At-05 | Africa | Atypical |
| TGp-At-06 | Africa | Atypical |
| TGp-At-07 | Africa | Atypical |
| TGp-At-08 | Africa | Atypical |

Table 5-2: Absorbance averages for each serum sample, for each peptide

| +/- | SERUM | 217 | 258 | 246 | 221 |
|---|---|---|---|---|---|
| POSITIVE | TGp-II-01 | 0,463 | 0,524 | 0,421 | 0,265 |
| | TGp-II-02 | 0,171 | 0,369 | 1,587 | 0,146 |
| | TGp-II-03 | 0,129 | 0,543 | 0,337 | 0,458 |
| | TGp-II-04 | 0,281 | 0,474 | 0,613 | 0,185 |
| | TGp-II-05 | 0,185 | 0,317 | 0,180 | 0,245 |
| | TGp-II-06 | 0,226 | 0,444 | 0,129 | 0,173 |
| | TGp-II-07 | 0,150 | 0,315 | 0,190 | 0,135 |
| | TGp-II-08 | 0,267 | 0,264 | 0,139 | 0,223 |
| | TGp-II-09 | 0,288 | 0,283 | 0,184 | 0,211 |
| | TGp-II-10 | 0,149 | 0,234 | 0,129 | 0,133 |
| | TGp-II-11 | 0,266 | 0,331 | 0,051 | 0,110 |
| | TGp-II-12 | 0,287 | 0,267 | 0,078 | 0,157 |
| | TGp-II-13 | 0,620 | 1,197 | 0,259 | 0,183 |
| | TGp-II-14 | 0,279 | 2,226 | 0,723 | 0,211 |
| | TGp-II-15 | 0,438 | 0,369 | 0,298 | 0,106 |
| | TGp-II-16 | 0,392 | 0,244 | 0,396 | 0,126 |
| | TGp-II-17 | 0,418 | 0,391 | 0,386 | 0,086 |
| | TGp-II-18 | 0,369 | 0,079 | 0,241 | 0,030 |
| | TGp-II-19 | 0,242 | 0,342 | 0,719 | 0,178 |
| | TGp-II-20 | 0,127 | 0,809 | 0,243 | 0,094 |
| | TGp-II-21 | 0,070 | 0,554 | 0,533 | 0,072 |
| | TGp-II-22 | 0,566 | 0,438 | 0,196 | 0,609 |
| | TGp-II-23 | 1,222 | 0,546 | 0,268 | 0,445 |
| | TGp-II-24 | 0,507 | 0,220 | 0,198 | 0,359 |
| | TGp-II-25 | 0,653 | 0,224 | 0,102 | 0,537 |
| | TGp-II-26 | 0,561 | 0,175 | 0,041 | 0,422 |
| | TGp-II-27 | 0,369 | 0,174 | 0,009 | 0,307 |
| | TGp-II-28 | 0,288 | 0,201 | 0,197 | 0,364 |
| | TGp-II-29 | 0,378 | 0,296 | 0,092 | 0,172 |
| | TGp-II-30 | 0,796 | 0,702 | 0,299 | 0,455 |
| | TGp-II-31 | 0,359 | 0,566 | 0,178 | 0,190 |
| | TGp-II-32 | 0,614 | 0,643 | 0,400 | 0,407 |

Table 5-2: Absorbance averages for each serum sample, for each peptide (cont.)

| +/- | SERUM | 217 | 258 | 246 | 221 |
|---|---|---|---|---|---|
| POSITIVE | TGp-I-01 | 0,680 | 0,653 | 0,315 | 0,874 |
| | TGp-I-02 | 0,744 | 0,815 | 0,269 | 1,057 |
| | TGp-I-03 | 0,484 | 0,290 | 0,171 | 0,317 |
| | TGp-I-04 | 0,360 | 0,277 | 0,336 | 0,257 |
| | TGp-I-05 | 0,442 | 0,487 | 0,357 | 0,385 |
| | TGp-I-06 | 0,347 | 0,337 | 0,274 | 0,472 |
| | TGp-I-07 | 0,316 | 0,243 | 0,087 | 0,264 |
| | TGp-I-08 | 0,215 | 0,376 | 0,134 | 0,260 |
| | TGp-I-09 | 0,328 | 0,526 | 0,188 | 0,373 |
| | TGp-I-10 | 0,219 | 0,238 | 0,148 | 0,295 |
| | TGp-I-11 | 0,387 | 0,221 | 0,172 | 0,986 |
| | TGp-I-12 | 0,275 | 0,119 | 0,123 | 0,387 |
| | TGp-III-12 | 0,564 | 0,368 | 0,142 | 0,867 |
| | TGp-III-01 | 0,125 | 0,140 | 0,098 | 0,292 |
| | TGp-III-02 | 0,252 | 0,118 | 0,019 | 0,692 |
| | TGp-III-03 | 0,861 | 0,868 | 1,235 | 0,604 |
| | TGp-III-04 | 0,191 | 0,151 | 0,515 | 0,407 |
| | TGp-III-05 | 0,368 | 0,229 | 0,441 | 2,605 |
| | TGp-III-06 | 0,680 | 0,268 | 0,446 | 1,867 |
| | TGp-III-07 | 0,448 | 0,169 | 0,384 | 0,340 |
| | TGp-III-08 | 0,406 | 0,141 | 0,322 | 0,776 |
| | TGp-III-09 | 0,400 | 0,126 | 0,370 | 0,293 |
| | TGp-III-10 | 0,491 | 0,211 | 0,394 | 0,574 |
| | TGp-III-11 | 0,235 | 0,143 | 0,208 | 0,232 |
| | TGp-At-01 | 0,456 | 0,224 | 0,548 | 0,444 |
| | TGp-At-02 | 1,126 | 0,403 | 0,783 | 0,674 |
| | TGp-At-03 | 0,564 | 0,205 | 0,548 | 0,323 |
| | TGp-At-04 | 0,158 | 0,125 | 0,129 | 0,281 |
| | TGp-At-05 | 0,887 | 0,618 | 0,466 | 0,944 |
| | TGp-At-06 | 0,641 | 0,736 | 0,472 | 0,644 |
| | TGp-At-07 | 0,456 | 0,587 | 0,381 | 0,444 |
| | TGp-At-08 | 0,257 | 0,379 | 0,247 | 0,475 |

Table 5-2: Absorbance averages for each serum sample, for each peptide (cont.)

| +/- | SERUM | 217 | 258 | 246 | 221 |
|---|---|---|---|---|---|
| NEGATIVE | TGn-01 | 0,193 | 0,502 | 0,485 | 0,760 |
| | TGn-02 | 0,296 | 0,385 | 0,710 | 0,583 |
| | TGn-03 | 0,526 | 0,163 | 0,129 | 0,470 |
| | TGn-04 | 0,667 | 0,694 | 0,101 | 0,581 |
| | TGn-05 | 0,024 | 0,455 | 0,193 | 0,748 |
| | TGn-06 | 0,315 | 0,402 | 0,116 | 0,268 |
| | TGn-07 | 0,101 | 0,197 | 0,267 | 0,757 |
| | TGn-08 | 0,091 | 0,182 | 0,212 | 1,135 |
| | TGn-09 | 0,258 | 0,571 | 0,214 | 0,341 |
| | TGn-10 | 0,228 | 0,306 | 0,077 | 0,336 |
| | TGn-11 | 0,378 | 0,276 | 0,244 | 0,455 |
| | TGn-12 | 0,286 | 0,299 | 0,344 | 1,092 |
| | TGn-13 | 0,332 | 0,254 | 0,081 | 0,690 |
| | TGn-14 | 0,284 | 0,188 | 0,226 | 0,228 |
| | TGn-15 | 0,694 | 0,938 | 0,057 | 0,482 |
| | TGn-16 | 0,207 | 0,223 | 0,162 | 0,386 |
| | TGn-17 | 0,253 | 0,373 | 0,280 | 0,216 |
| | TGn-18 | 0,175 | 0,111 | 0,131 | 0,265 |
| | TGn-19 | 0,359 | 0,359 | 0,463 | 0,593 |
| | TGn-20 | 0,487 | 0,292 | 0,463 | 0,270 |

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