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SEROTYPING OF *TOXOPLASMA GONDII*

CONTRIBUTIONS TO THE KNOWLEDGE OF PARASITE BIODIVERSITY

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Avó

“Cessem do sábio Grego e do Troiano

As navegações grandes que fizeram;

Cale-se de Alexandro e de Trajano

A fama das vitórias que tiveram;

Que eu canto o peito ilustre Lusitano,

A quem Neptuno e Marte obdeceram.

Cesse tudo o que a Musa antiga canta,

Que outro valor mais alto se alevanta.”

(Luís de Camões, in “Os Lusíadas”, canto I)

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Abstract

Toxoplasmosis is caused by the Apicomplexa parasite *Toxoplasma gondii*. Most infections in humans are asymptomatic. However, severe complications may occur in consequence of a congenital infection or in immunocompromised patients due to an acquired infection. Outbreaks of human toxoplasmosis have been described, where immunocompetent individuals developed severe infections. Isolation of parasite associated with these clinical manifestations from different geographic regions revealed that *T. gondii* has a high genetic diversity. Most of the methods used for genetic characterization of this parasite, require the previous isolation of the parasite or of the DNA. This limits the genetic studies to infections from which is possible to collect biological samples, usually from symptomatic patients. Besides, isolation by mice bioassay may favour some strains and do not detect mixed infections.

Serotyping is a simple typing method that consists of an immunoenzymatic assay (enzyme-linked immunosorbent assay [ELISA]) using synthetic polymorphic peptides derived from *T. gondii* antigens. The only biological material required is serum samples, which can be collected from both symptomatic and asymptomatic patients and eliminates the biases associated with genotyping based on parasite isolation. The main objective of this study is to explore new polymorphic strain-specific peptides for serotyping. Sequencing of the coding genes for three antigens (GRA6, GRA7 and GRA8) from 52 archetypal and non-archetypal strains showed that GRA6 and GRA7 had polymorphisms that could be used to differentiate the three clonal lineages and non-archetypal strains. Serotyping of infections in mice and humans with known strains showed that infections due to strains belonging to the archetypal lineages I, II and III can be serotyped with the peptides GRA6II, GRA6I/III and GRA7III. However, peptides specific for non-archetypal strains have a poor specificity or sensitivity. This method was also used to predict the serotype of patients with acute and chronic toxoplasmosis due to unknown genotypes from Europe, Africa, and Latin America. In Europe the prevalent serotype is type II, while in Africa and Latin America, serotype GRA6I/III prevails. Serotype II was found in Uruguay with a considerable frequency in areas with possible influence of genotypes until now described in Europe. No relation was established between serotype distribution and pathology. Serotyping of naturally infected domestic

animals presented some limitations with the selected peptides. The results suggest that although the actual limitations, serotyping may be a promising method for typing strains. However, other peptides from different markers must be studied in order to discriminate archetypal from non-archetypal strains. In the future, serotyping may be valuable tool in epidemiologic studies, in the detection of a re-infection with a different strain and also in the detection of *T. gondii* infections by non-archetypal strains in animals for human consumption.

Keywords : *Toxoplasma gondii* ; genotypes; serotyping ; GRA6 ; GRA7 ; polymorphic peptides

Résumé

"Sérotypage de *Toxoplasma gondii* - Apport à la connaissance de la biodiversité du parasite"

La toxoplasmose est une maladie causée par le parasite apicomplexe *Toxoplasma gondii*. La plupart des infections humaines sont asymptomatiques, cependant, de sévères complications peuvent se développer à la suite d'une infection congénitale ou chez des patients immunodéprimés. Des foyers de toxoplasmose, dans lesquels des individus immunocompétents développent des infections sévères, ont été décrits dans la littérature. L'isolement du parasite associé à ces manifestations cliniques de différentes régions géographiques a révélé la grande diversité génétique de *T. gondii*. Les méthodes utilisées pour la caractérisation génétique de ce parasite nécessitent l'isolement préalable du parasite ou de son ADN. Cette condition réduit les études génétiques aux infections de sujets symptomatiques, dans lesquelles il est possible de recueillir des échantillons biologiques. D'autre part, l'isolement par bioessais sur souris peut favoriser certaines souches et ne pas permettre la détection d'infections mixtes.

Le sérotypage est une méthode simple basée sur un essai immunoenzymatique (*enzyme-linked immunosorbent assay* – ELISA) qui utilise des peptides polymorphes dérivés d'antigènes de *T. gondii*. Les sérums, seuls échantillons biologiques nécessaires, peuvent être obtenus de sujets présentant ou non des symptômes et éliminent les disparités associées au génotypage à partir de l'isolement du parasite. Le principal objectif de ce travail de thèse est d'étudier l'applicabilité au sérotypage de nouveaux peptides polymorphes souches-spécifiques. Le séquençage des gènes codant pour trois antigènes (GRA6, GRA7, GRA8) de 52 souches archétypales et non-archétypales a montré que les peptides GRA6 et GRA7 présentent des polymorphismes capables de distinguer les trois lignées clonales et les souches non-archétypales. Le sérotypage de souches connues de souris et d'humains infectés a montré que les infections dues à des souches appartenant aux lignées archétypales I, II et III peuvent être sérotypées avec les peptides GRA6II, GRA6I/III et GRA7III. Toutefois, les peptides spécifiques de souches non-archétypales ont une faible spécificité et sensitivité. Cette méthode a également été utilisée pour prévoir le sérotype chez des patients atteints de toxoplasmose aiguë ou chronique dues à des génotypes inconnus d'Europe, d'Afrique, et d'Amérique Latine. En Europe, le sérotype II prédomine, alors qu'en Afrique et en Amérique Latine, le sérotype I/III est plus fréquent. Le sérotype II a cependant été retrouvé avec une

fréquence non négligeable en Uruguay, dans des zones où l'influence sur la circulation des génotypes des relations avec l'Europe est possible. Aucune relation n'a été établie entre la distribution des sérotypes et la pathologie. Les résultats du sérotypage d'animaux domestiques naturellement infectés avec les peptides sélectionnés, suggèrent malgré quelques restrictions, que le sérotypage est une méthode prometteuse pour certaines souches. D'autres peptides de différents marqueurs doivent néanmoins être étudiés afin de distinguer les souches archétypales des non-archétypales. Le sérotypage peut devenir un outil intéressant dans des études épidémiologiques, dans la détection d'une réinfection avec une souche différente, mais également dans la détection d'infections par des souches non-archétypales de *T. gondii* chez des animaux destinés à la consommation humaine.

Mots-clé: *Toxoplasma gondii* ; génotypes ; serotypage ; GRA6 ; GRA7 ; peptides polymorphes

Resumo

"Serotipagem de *Toxplasma gondii* – contributo para o conhecimento da biodiversidade do parasita"

A toxoplasmose é causada pelo parasita Apicomplexa *Toxoplasma gondii*. No homem, as infecções são habitualmente assintomáticas. No entanto, quadros clínicos graves podem ocorrer em consequência de uma infecção congénita ou em indivíduos immunodeprimidos. A toxoplasmose severa em doentes immunocompetentes, com quadros clínicos inabituais tem sido descrita em regiões da América do Sul. A caracterização genética das estirpes de *Toxoplasma gondii* associadas a estes casos revelou a existência de estirpes não arquétipos. A diversidade genética deste parasita é na realidade maior do que os arquétipos I, II e III previamente descritos. A caracterização genética do parasita requer o isolamento prévio do parasita ou do seu ADN, o que limita os estudos genéticos às infecções sintomáticas, onde é possível obter amostras biológicas. O isolamento do parasita por bioensaio em ratinhos pode favorecer certas estirpes em detrimento de outras, no caso de infecções mistas.

A serotipagem é um método simples, que tem por base um ensaio imunoenzimático (*enzyme-linked immunosorbent assay* – ELISA) usando, como antigénio, péptidos sintéticos polimórficos desenhados a partir de genes de *T. gondii*. Neste método, os soros são as únicas amostras biológicas necessárias, que podem ser obtidas quer de doentes sintomáticos como assintomáticos. O principal objectivo deste trabalho é desenvolver novos péptidos polimórficos específicos das diferentes estirpes, e que permitam uma efectiva discriminação na caracterização dos genotipos infectantes. A sequenciação da região codificante para três antigénios (GRA6, GRA7 e GRA8) de 52 estirpes arquétipos e não arquétipos demonstrou que os péptidos GRA6 e GRA7 têm polimorfismos capazes de distinguir as três linhas clonais, I , II e III das estirpes não arquétipos. A serotipagem de ratinhos e humanos infectados com estirpes conhecidas demonstrou que as infecções provocadas por estirpes de genotipo I, II e III podem ser serotipadas com os péptidos GRA6II, GRA6I/III e GRA7III. Os péptidos específicos das estirpes não arquétipos evidenciaram uma baixa especificidade e sensibilidade. Este método foi também usado para avaliação predictiva de serotipos em doentes com toxoplasmose aguda e crónica, da Europa, Africa e América Latina, nos quais se

desconhecia o genotipo infectante. Na Europa, o serotipo II predomina, enquanto em Africa e na América Latina, o serotipo I/III é mais frequente. O serotipo II foi encontrado com uma frequência considerável no Uruguai. Não se estabeleceu nenhuma relação entre o serotipo e a patologia. Os resultados da serotipagem de animais domésticos naturalmente infectados, apesar de algumas limitações, sugerem que este pode ser um método promissor. Outros péptidos de diferentes marcadores que permitam a diferenciação das estirpes arquétipos das não arquétipos têm de ser estudados. No futuro, a serotipagem pode tornar-se um instrumento importante em estudos epidemiológicos, na detecção de re-infecções com estirpes diferentes, bem como na detecção de infecções por estirpes não arquétipos em animais destinados ao consumo humano.

Palavras chave: *Toxoplasma gondii*; genotipo; serotipagem; GRA6; GRA7; péptidos polimorficos

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Abbreviations

aa – Aminoacids

ADP – Adenosine Diphosphate

AIDS – Adquired Immunodeficiency Syndrome

BSA – Bovine Serum Albumin

CAMLG – Calcium Modulating Ligand

CDC – Center for Disease Control and Prevention

DNA – Desoxyribonucleic acid

ELISA – Enzyme Lynked Immunosorbent Assay

GRA – Granule Dense Antigen

GST – Glutathione-S-Transferase

HAART – Highly Active Antirectroviral Therapy

HIV – Human Immunodeficiency Virus

IFAT – Immunofluorescence Antibody Test

Ig – Immunoglobulin

MAT – Modified Agglutination Test

MIC – Micronemes

MS – Microsatellites

NTPase – Nucleotide Triphosphate Hydrolase

OD – Optical Density

OPD – O-Phenylenediamine

PBS – Phosphate Buffer Saline

PCR – Polimerase Chain Reaction

PV – Parasitophorous Vacuole

PVM – Parasitophorous Vacuole Membrane

RFLP – Restriction Fragment Length Polymorphism

SAG – Surface Antigen

SNP – Single Nucleotide Polymorphism

SRS – SAG1 related sequence

T. gondii – Toxoplasma gondii

TE – Toxoplasmic Encephalitis

Aminoacids

A – Alanine

D – Aspartic acid

E – Glutamic acid

F – Phenylalanine

G – Glycine

H – Histidine

I – Isoleucine

K – Lysine

L – Leucine

M - Methionine

N – Asparagine

P – Proline

Q – Glutamine

R – Arginine

S – Serine

T – Threonine

V – Valine

Y – Tyrosine

Nucleotides

A – Adenine

C – Cytosine

G – Guanine

T – Thymine

Introduction

Infections by the Apicomplexa protozoa *Toxoplasma gondii* are widespread in humans and other warm-blooded animals. *T. gondii* is the only species in the genus. It is capable of infections not only in an unusually wide range of hosts but also in many different host cells. The life cycle of the parasite includes an asexual reproduction in the intermediate hosts (mammals and birds) and a sexual reproduction in the definitive host (Felidae). Ingestion of environmental oocysts (by drinking water or eating raw vegetables), eating raw or undercooked meat containing tissue cysts stages are the main transmission routes in humans and animals (Carme *et al*., 2002; de Moura *et al*., 2006; Heukelbach *et al*., 2007). Although toxoplasmosis is the most reported parasitic zoonosis in Europe it is believed that the incidence of human disease and the parasite occurrence in animals, food and water is underestimated (EFSA, 2007). Since the discovery of a *T. gondii* specific antibody test, the Sabin-Feldman dye test, the real importance of the disease in medical and veterinary fields is growing. However, hygiene remains the best measure to prevent *Toxoplasma* infection in humans (Dubey, 2008).

Recently, severe cases of toxoplasmosis have occurred specially in South America. The genetic characterization of *T. gondii* associated with these cases revealed the emergence of non archetypal genotypes strains other than those previously described archetypal lineage I, II, and III as population structure of *Toxoplasma.* Data from many parts of the world suggests a different geographical distribution of the genotypes and also differences in clinical presentation associated-parasites. A postulate has emerged in authors mind: the possible correlation between the pathogenesis of toxoplasmosis and the genetic characteristics of the disease-associated strains. Until now, there are no sufficient evidences to sustain a clear correlation between strain genotypes and human or animal disease. In order to a better comprehension of the pathogenesis of toxoplasmosis it is necessary to proceed to the genetic characterization of *T. gondii* associated with chronic and acute infections.

The previously defined strategy based on bioassay for the isolation of parasites and subsequent multilocus study polymorphisms (SAG2 and microsatellite) is laborious and time consuming. Other considerable limitation is recognised, besides the need of the parasite or

DNA: in the case of mixed infections, in a particular host, it may favour the isolation of one strain, restringing others.

Recently several authors have hypothesised serotyping for genetic characterization of *T. gondii* strains. Serotyping is a typing method based on the antibody recognition of strain-specific polymorphic peptides. The aim of this work was to study the applicability of the same procedure for serotyping serum samples provided from human and animal infections. We have used a new ELISA test based on GRA6, GRA7 and GRA8 C-terminal polymorphic peptides. Our results and others published by two other scientific teams suggest that serotyping is a very promising method for typing *T. gondii* strains in the future.

Part I

Literature review

1.1 The parasite *Toxoplasma gondii*

1.1.1 History

Toxoplasma gondii was first described in 1908 by Nicolle and Manceaux, in mononuclear cells of the spleen and liver of a *Ctenodactylus gundi* (a rodent from North Africa). In the same year in Brazil, Splendore isolated it from a rabbit that had died with paralysis. The name *Toxoplasma* comes from the Greek "toxon", which means arc. The first case in humans associated to *T. gondii* was described in 1923 by Janku in Prague in the retina of a child with congenital infection (hydrocephalus and microphtalmia). In consequence, Levaditi in 1928 suggested an association between congenital hydrocephalus and toxoplasmosis. Wolf and Cowen in 1937 described a fatal case of infantile granulomatous encephalitis that Sabin and Olitski related to *Toxoplasma*. Based on their studies, Wolf and Cowen established that *T. gondii* was a cause of congenital disease. In 1940, Pinkerton and Weinman, described for the first time an acquired case of toxoplasmosis in a young man. In 1948, Sabin and Feldman implemented the dye test, the most specific serological test for toxoplasmosis for human infection. The classical tetrad of symptoms of congenital infection (retinochoroiditis, hydrocephalus or microcephalus, cerebral calcification and psychomotor disturbances) was described in 1952. Only in 1969, the cat was found to be the definitive host, when Hutchison found oocysts in feline feces. In 1970, the life cycle of *T. gondii* was completely defined with the description of the sexual phase of the life cycle in the small intestine of cats. The first cases of cerebral toxoplasmosis in AIDS patients were described in 1981-82 (reviewed by Dubey and Beattie, 1988; reviewed by Tenter *et al.*, 2000).

1.1.2 Taxonomy

Toxoplasma gondii belongs to the kingdom Protista, subkingdom Protozoa, phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Eucoccidiorida, family, Sarcocystidae, genus *Toxoplasma* and species *gondii*.

1.1.3 Life cycle

T. gondii has a heteroxenous life cycle. Sexual development occurs exclusively in the definitive hosts, which are members of the family *Felidae* (ex: domestic cats). Asexual development occurs in both definitive and intermediate hosts. Warm-blooded animals including man can be *Toxoplasma* intermediate hosts (reviewed by Tenter *et al*., 2000).

There are four stages in the life cycle of *T. gondii*: tachyzoites, merozoites, bradyzoites (in tissue cysts) and sporozoites (in oocysts). Tachyzoites, bradyzoites and sporozoites are infectious for both intermediate and definitive hosts (Dubey *et al*., 1998), which may acquire a *T. gondii* infection mainly via one of the following routes (Figure 1): horizontally, by oral ingestion of infectious oocysts from the environment; horizontally, by oral ingestion of tissue cysts contained in raw or undercooked meat; or vertically, by transplacental transmission of tachyzoites. Thus *T. gondii* may be transmitted from definitive to intermediate hosts, from intermediate to definitive hosts, as well as between definitive and between intermediate hosts (reviewed by Tenter *et al*., 2000).

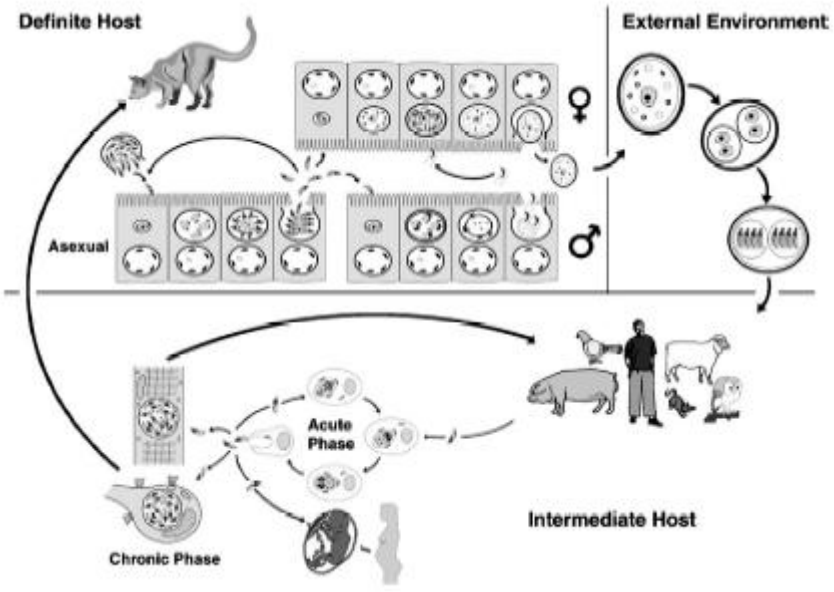


Figure 1: *Toxoplasma gondii* life cycle (From Ferguson, 2004).

The life cycle of *T. gondii* in the definitive host differs from that in the intermediate hosts (Figure 1). In intermediate hosts, *T. gondii* undergoes two phases of asexual

development. In the first phase, tachyzoites multiply rapidly by repeated endodyogeny in many different types of host cells. In a second phase, tachyzoites undergo stage conversion to initiate bradyzoite development, which results in the formation of tissue cysts. Within the tissue cyst, bradyzoites multiply slowly by endodyogeny. Tissue cysts are located predominantly in the central nervous system, the eye as well as skeletal and cardiac muscles. Tissue cysts are the terminal life-cycle stage in the intermediate host and are immediately infectious. If ingested by a definitive host, the bradyzoites initiate another asexual phase of proliferation (merozoites), which consists of initial multiplication by endodyogeny followed by repeated endopolygeny in epithelial cells of the cat small intestine. Merozoites proliferation is limited to a few (2-4) generations within the enterocytes of the cat gut where it gives rise to the sexual (microgametocytes and macrogametocytes) stages. The terminal stages of this asexual multiplication initiate the sexual phase of the life cycle. Gamogony and oocyst formation take place in the epithelium of the cat small intestine. Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the feces. Sporogony occurs outside the host and leads to the development of infectious oocysts, which contain two sporocysts, each containing four sporozoites (reviewed by Dubey *et al*., 1998; reviewed by Tenter *et al*., 2000).

The role of bradyzoites and sporozoites is to transmit infection between hosts and as such they have to survive the rigors of the external environment and/or the digestive tract of the new host. Sporozoites and mature bradyzoites are non-replicative stages (Radke *et al*, 2003).

1.1.3.1 Tachyzoite

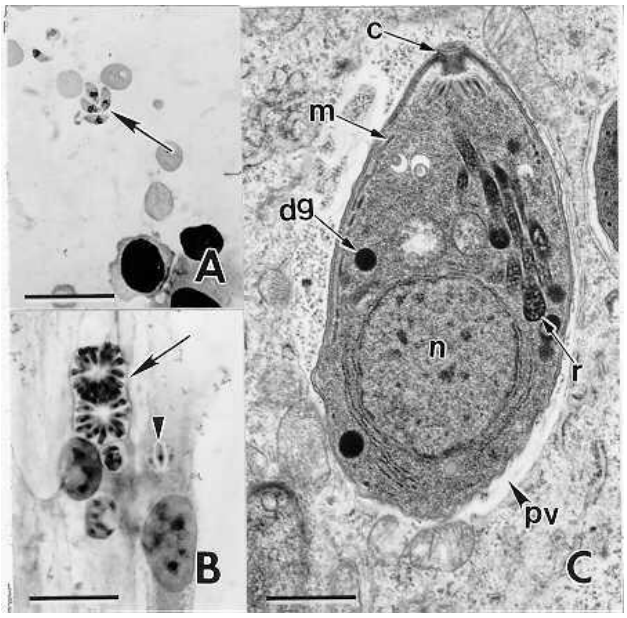


Figure 2: Tachyzoites of *T. gondii*. A. Extracellular (arrow) released from host cells. B. Intracellular in cell culture. Note a group arranged in a rosette (arrow) and vacuole (arrowhead) around a tachyzoite. C. Transmission electron micrograph of an intracellular tachyzoite. Note a parasitophorous vacuole (PV) around the tachyzoite. Parasite organelles visible in this picture include a conoid (c), micronemes (m), dense granules (dg) nucleus (n) and rhoptries (r), (From Dubey, http://gsbs.utmb.edu/microbook/ch084.htm).

Tachyzoites have a crescent shape with 2 x 6μm. This stage has a high rate of multiplication and requires an intracellular habitat to survive and multiply. Tachyzoites enter the cells by direct penetration or by phagocytosis and multiply by endodyogeny within the host cell. The tachyzoites are associated to the acute phase of infection, during which they invade host cells. After invasion, the parasite multiplies and form rosettes (Figure 2B). Cell cytoplasm becomes filled with parasites leading to cell disruption, upon which the released tachyzoites invade contiguous cells by active invasion of the host cell membrane or by phagocytosis.

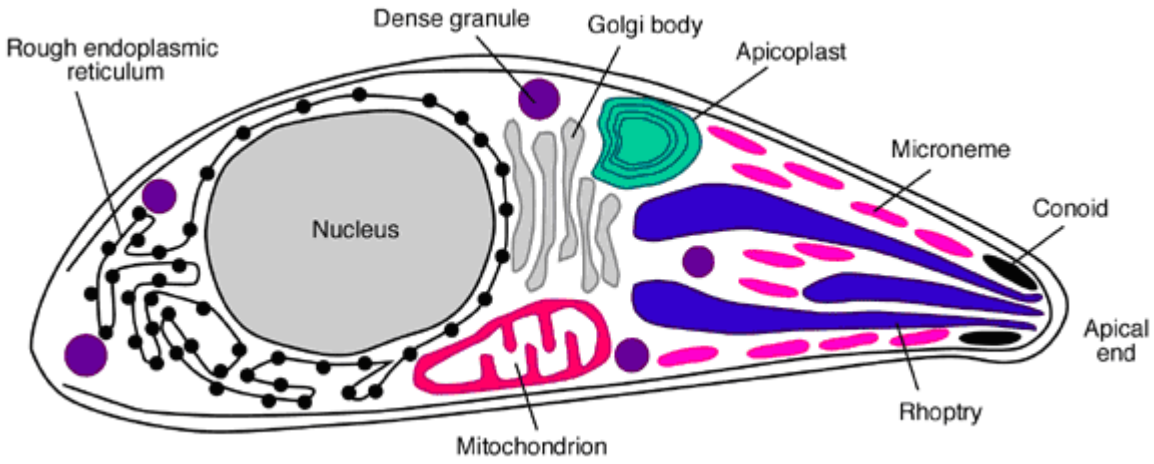


Figure 3: Ultrastructure of a *Toxoplasma gondii* tachyzoite (From Ajioka, Fitzpatrick and Reitter, www-ermm.cbcu.cam.ac.uk/01002216h.htm).

Tachyzoites have a pellicle, subpellicular microtubules, a polar ring, a conoid, rhoptries, micronemes, mitochondria, endoplasmatic reticulum, Golgi apparatus, ribosomes, rough surface endoplasmatic reticulum, micropores and a centrally located nucleus. The conoid, the rhoptries and the micronemes are characteristic structures of this parasitic form (Figures 2C and 3). The pellicle consists of three membranes. The inner membrane is discontinuous in three areas, at the polar ring (anterior), at the micropore (lateral), and the posterior end. The polar ring is an osmiophilic thickening of the inner membrane at the anterior end of the tachyzoite. The conoid is located at the polar end. It is a cylindrical cone which consists of six to eight fibrillar elements arranged like a compressed spring. This structure is probably associated with the penetration of the tachyzoite through the membrane of the host cell. Terminating within the conoid are the rhoptries. These are four to ten gland-like structures with an anterior narrow neck and posterior-sac-like end reaching as far as the nucleus. The rhoptries have a secretory function associated with host cell penetration. When the parasite has attached to the host cell, their contents are discharged through the conoid. The micronemes are tube like structures at the anterior end of the organism. They are usually fewer than 100 situated at the conoidal end of the parasite and are also involved in invasion of the host cell. The parasite also contains dense granules that secret the dense granule proteins. The conoid can rotate, extend and retract and is important when the parasite searches for an attachment site at the host cell. The micropores are sites specialized for the uptake of nutrients

through endocytosis. After entry into the host cell, the parasite is surrounded by a parasitophorous vacuole membrane (PVM). *Toxoplasma gondii* enters the host cell by active invasion (Dubey *et al*., 1998).

1.1.3.2 Bradyzoite and tissue cysts

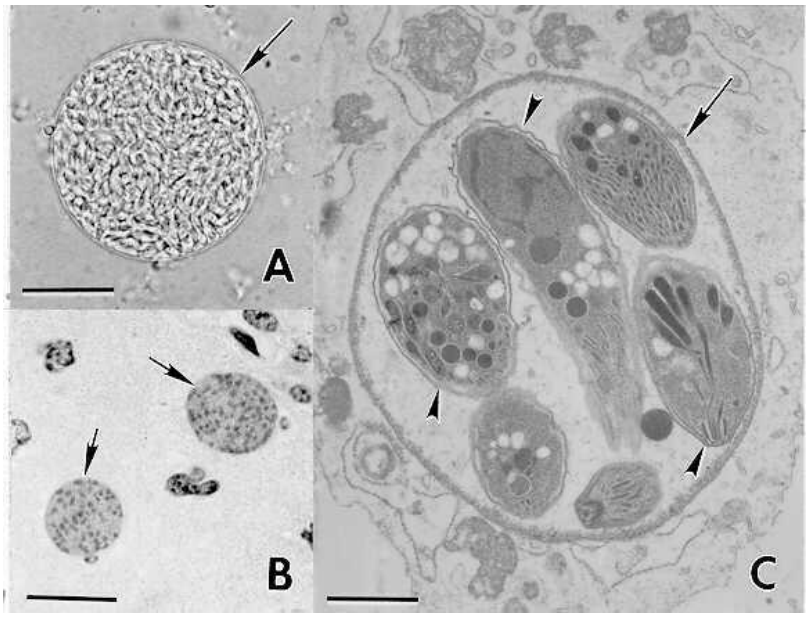


Figure 4: Tissue cysts of *T. gondii*. A. Tissue cyst freed from mouse brain. Note a thin (arrow) cyst wall enclosing hundreds of bradyzoites. B. Two tissue cysts (arrows) in section of brain. C. Transmission electron micrograph of a small tissue cyst in cell culture. Note thin cyst wall (arrow) enclosing 6 bradyzoites (arrowheads), (From Dubey, http://gsbs.utmb.edu/microbook/ch084.htm).

Structurally, bradyzoites slightly differ from tachyzoites. The nucleus is located toward the posterior end, whereas in tachyzoites is more central. Most bradyzoites have one to three rhoptries, which are looped back on them. Bradyzoites contain several amylopectin granules, which are usually absent in tachyzoites.

The cysts are formed within the host cell and may vary in size from cysts with only few bradyzoites to cysts with a large number of organisms (Figure 4). The tissue cyst develops and remains in the host cell cytoplasm, and its wall is intimately associated with the host cell endoplasmic reticulum and mitochondria. The tissue cyst contains both host-derived

and *T. gondii* antigens. Tissue cysts vary in size from 5 to 60 μm in the brain and 100 μm in other tissues. The cyst wall has less than 0.5 μm. Brain, eye, heart and skeletal muscle are the most common sites for cyst location. Cysts are spherical in the brain and conform to the shape of muscle fibres in heart and skeletal muscles. These parasitic forms are not resistant to freezing and thawing, heating above 66ºC and desiccation. However, they can survive as long as 2 months at 4ºC. The factors determining tissue cyst rupture are unknown.

1.1.3.3 Oocyst

Unsporulated oocysts are subspherical to spherical with 10x12 μm in diameter. Sporulated oocysts are subspherical to ellipsoid with 11x13 μm in diameter. Each sporulated oocyst contains two sporocysts measuring 6x8 μm. Each sporocyst divides into 4 sporozoites, measuring 2 x 6 to 8 μm. Thus, the sporulated oocysts contain eight sporozoites. Oocyst wall of sporolated oocysts consist of three layers. One electron-dense outer layer, a electron-lucent middle layer and a moderately electron-dense inner layer (reviewed by Dubey *et al*., 1998). Oocysts sporulation depends on the temperature and oxygen and may take 1 to 21 days. Sporulation takes place in 2 to 3 days at 24ºC, 5 to 8 days at 15ºC and 14 to 21 days at 11ºC. Sporulation does not occur below 4ºC or above 37ºC. UV treatment can be an effective disinfection method to inactivate *T. gondii* oocysts in drinking water (Dumètre *et al*., 2008). Continuous UV exposure is very effective to inactivate >99.9% of *T. gondii* oocysts.

In summary, *Toxoplasma gondii* has a worldwide distribution and is capable of infect a wide range of hosts and also many different host cells. The multiple biological stages, their virulence and the transmission rotes between the different hosts (definitive and intermediate) are responsible for the success of life maintenance of this parasite all over the world.

1.2 Epidemiology

Seroprevalence of *Toxoplasma gondii* is highly variable among different geographic regions. Even for the same continent, deep variations can be found for the different countries. Several reasons may explain this fact such as: diet, preparation of food, hygiene, environmental conditions, definitive host population (wild and domestic Felidae) and different laboratory techniques used for serodiagnosis.

Seroprevalence data in humans are limited to certain geographical areas. In Europe, a lower prevalence was found in the north countries such as Scandinavia (Norway, 10.9%; Swede, 18%; Finland, 20.3%; Denmark, 27.8%). In south and center countries, prevalence is higher and varies from 30% in Spain to 77.4% in Yugoslavia (AFSSA, 2005). In France, seroprevalence in 2003 was 44%. In Portugal, seroprevalence was 35% in the south and 65% in the north (EFSA, 2007). North America prevalence is usually less than 30%. On the contrary, in South America, prevalence is usually more than 50% (AFSSA, 2005). Seroprevalence is also high in Africa ranging from 18 to 83.5% (AFSSA, 2005; Petersen, 2007; Tenter *et al*., 2000). In Asia the seroprevalence varies from 4 to 55.7% (AFSSA, 2005; Petersen, 2007; Tenter *et al*., 2000).

Several seroprevalence studies from different geographic origins and in different hosts (domestic and wild animals) are referred in appendix 1.

In summary, seroprevalence evaluates the exposition rate to *T. gondii* in a given population. However, these studies do not give the real prevalence of toxoplasmosis, since they are limited to a specific population and region.

1.3 Toxoplasmosis in humans

Toxoplasmosis in immunocompetent people is usually asymptomatic. Although not very common, clinical signs may be associated to an acute *Toxoplasma* infection in immunocompetent persons. Severe forms of *Toxoplasma* infection are mainly associated with congenital infections, as a result of a primo-infection of the mother and with immunocompromised patients, also as consequence of a primo-infection or a reactivation of a chronic infection. In immunocompetent patients, severe cases of toxoplasmosis have been described associated to highly virulent non-archetypal *Toxoplasma* strains.

1.3.1 Congenital infection

Congenital infection results from a primary infection of the mother during pregnancy. *Toxoplasma gondii* infection acquired during pregnancy varies from subclinical to severe foetal infection, which may result in foetal loss or several lesions involving mainly the brain and eye. Other organs such as liver, spleen, kidneys and lungs may also be involved. Sabin described the tetrad of clinical symptoms of severe congenital infection: microcephaly or macrocephaly, intracranial calcification, psycomothors disturbs and chorioretinitis (reviewed by Lopes *et al*., 2007). The severity of foetal damage mainly depends on the stage of pregnancy at the time of infection. The foetal infection rate is higher when the maternal infection occurs later in pregnancy, but the severity is lower (Table 1), because immunologic maturation had time to develop. Parasite concentration in the amniotic fluid is also related with severity of infection (Romand *et al*., 2004)

Table 1: Risk of congenital infection and relation between clinical manifestations in the newborn and maternal infection (Thulliez and Romand 2001)

Mother infection (weeks)	Rate of transplacental transmission (%)	Clinical signs in the newborn (%)
13	6	61
26	40	25
36	72	9

The risk of foetal lost or disease is higher if infection occurs during early pregnancy. Infection during late pregnancy is often subclinical in the newborn. Although these infants appear healthy at birth, they may develop clinical symptoms later in life, predominantly with ocular involvement. Chorioretinal scars are the most frequent reported ophthalmologic manifestations of congenital infection (reviewed by Mets and Chhabra, 2008). Treatment of congenital infections during the first year of life seems important in prevention of new ocular lesions. However, new chorioretinal lesions, even in treated children can occur at the age of ten or older (Phan *et al*., 2008).

1.3.2 Toxoplasmosis in immunocompromised patients

Opportunistic infection by *T. gondii* in immunocompromised patients (AIDS and transplanted patients) with a high deficit of T cells (<100 cells/ml), can reactivate an acquired latent infection, which may be fatal if not recognized and treated early. In these patients, the more frequent lesions appear in the brain, but other organs like eye, lung, and heart (Dixit *et al*., 2007) can also be affected. Toxoplasmic encephalitis (TE) and disseminated toxoplasmosis have been observed in immunocompromised patients due to HIV infection, transplantation of organs or bone marrow (due to an organ from infected donor or from reactivation of a latent infection in the recipient due to immunosuppressive treatment), Hodgkin's disease or immunosuppressive therapy. It is estimated that in up to 40% of AIDS patients, *Toxoplasma* is the cause of severe encephalitis and 10-30% of AIDS patients infected with *Toxoplasma* die due toxoplasmosis (Tenter at al., 2000). Highly active

antiretroviral therapy (HAART) has been responsible for the decrease of *Toxoplasma* encephalitis rate in AIDS patients. Cerebral involvement is found more frequently in AIDS cases, while pulmonary involvement is found mainly in non-HIV immunocompromised patients (Ajzenberg *et al*., 2009). Cases of cerebral toxoplasmosis were reported in solid organ transplants (mainly heart, but also kidney), in peripheral stem cells and allogeneic bone marrow transplanted patients (Abecassis, 1991; Cibickova *et al*., 2007; da Cunha *et al*., 1993). A retrospective study of the Fukuoka BMT group revealed only two cases of TE (one fatal) in 925 allogeneic hematopoietic stem cell transplantations during a period of 17 years (Matsuo *et al*., 2007). Three main risk factors were defined for the outcome of *Toxoplasma* infection in immunocompromised patients: no specific treatment, a pulmonary involvement and a disseminated toxoplasmosis (Ajzenberg *et al*., 2009). Computed tomography and magnetic resonance imaging are useful tools in the diagnosis of TE. Using these imaging techniques, the spherical regions of tissue necrosis resulting from focal parasite growth from the initial site of a tissue cyst are often visible. Multiple lesions are frequently seen in TE patients, most commonly in the cerebral hemispheres and basal ganglia (Strittmatter *et al*., 1992). Imaging diagnosis of TE remains a serious challenge because TE lesions can be difficult to distinguish from lymphomas or bacterial abscesses and direct detection of the parasite in tissues is rarely performed due to the inherent dangers of brain biopsies (Carruthers, 2002).

1.3.3 Toxoplasmosis in immunocompetent patients

Toxoplasmosis in immunocompetent people is usually asymptomatic. Although not very common, clinical signs usually associated to an acute infection in immunocompetents persons are adenopathies, fever, headache and fatigue. Rarely, immunocompetent patients may present an acute *Toxoplasma* infection with multivisceral involvement. Bilateral pneumopathy and hepatic cytolysis were the most frequent pathologies. Neurological disorders (loss of consciousness), echographic cardiac abnormalities (mild dilated myocardiopathy with pericarditis), myositis, renal dysfunction, diarrhea, retinochoroiditis can be associated. Death can occur (Demar *et al*., 2007). Most of these cases of severe primary toxoplasmosis were reported in French-Guiana in immunocompetent adults. These cases were associated with the consumption of game (Carme *et al*., 2002). In Brazil, there is also a report of an immunocompetent individual who developed a pneumonia caused by *T. gondii* (Leal *et*

al., 2007). Ocular lesions are also frequent in immunocompetent individuals with *T. gondii* infection.

1.3.4 Psychiatric disorders

An association between *Toxoplasma* infection and schizophrenia has been suggested. A significant positive association was found between *T. gondii* IgG antibody and schizophrenia in several studies of *T. gondii* antibodies among individuals with a diagnosis of schizophrenia (Cetinkaya *et al*., 2007; Hinze-Selch *et al*., 2007; Niebuhr *et al*., 2008). The antibody titer among schizophrenia patients is significantly higher than in healthy patients. Behavior changes due to *Toxoplasma* infection was also demonstrated in animals. Rats infected with *Toxoplasma* lose their ability to avoid cats and consequently increase the chances of being eaten by the cat (Webster, 2007). Valproic acid, a drug used in patients with schizophrenia was shown to inhibit the growth of *T. gondii in vitro* (Jones-Brando *et al*., 2003).

In summary, expression of toxoplasmosis is mainly dependent of host immunity and genetic background. Congenitally infected children and immunocompromised patients are usually associated with severe infections. In immunocompetent patients, severe toxoplasmosis is usually associated with highly virulent strains.

1.4 Laboratory diagnosis

The diagnosis of toxoplasmosis is based on serological methods, and on the direct detection of the parasite or its DNA.

The study of the kinetic of the different immunoglobulins, mainly IgG and IgM, allow identifying five serological phases. In a first phase, no IgG or IgM is detected. The serology remains negative at this phase between contamination and beginning of humoral specific response. It lasts between 8-10 days. In a second phase only IgM is detected. At a third phase, IgM still remains and IgG starts to appear. The appearance and increase in IgG titer confirms the existence of an infection by *T. gondii*. This period varies between 2 to 6 months post-infection. In a fourth phase, the IgG titer raises to its high value, associated with IgM presence. The fifth phase, which corresponds to an infection more than 6 months old, is characterized by the presence of IgG and not existence of IgM.

In the newborn, a serological follow-up should be performed if an acute infection in the mother is detected during pregnancy. IgG antibodies in the sera of the newborn can be originary from the baby or acquired from the mother through the placenta. IgG antibodies inherited from the mother decrease and disappear at from 6-12 months of age, whereas self-IgG in the newborn persist or increases. IgM and IgA do not cross the placenta and their presence usually indicates a congenital infection (reviewed by Lopes *et al*., 2007; Petersen, 2007). IgG subclass analysis of serum samples from mother and child against defined recombinant antigens may improve diagnosis of congenital infection in newborns. The newborn primarily produces IgG2 and IgG3, whereas the maternally transferred antibodies are primarily IgG1 (reviewed by Petersen, 2007).

Serologically, the persistence of IgG or the appearance of specific IgM in the newborn and the isolation of the parasite from placenta, cord blood or amniotic liquid are indicative of a possible congenital infection.

Toxoplasma-specific antibodies can be detected in serum samples and cerebrospinal fluid in AIDS patients with suspicion of cerebral involvement. According to CDC (USA

Federal Centers for Disease Control and Prevention) criteria, serology is not a good tool for the diagnosis of TE, especially in immunocompromised patients with $CD4^+$ cell count less than 200. However, some antigens of medium and small molecular weight detected by Western-blot have been described has being important for diagnosis (Leport *et al*, 2001).

Ocular toxoplasmosis can be diagnosed by performing paired serological test for *Toxoplasma* antibodies in the eye and peripheral blood and thereby determining intraocular pathogen-specific antibody production, which is an indirect proof of intraocular *Toxoplasma* infection (Kijlstra *et al*., 1989). The anti-IgG specificity in the ocular fluid differed from the antigen specificity in the sera in chronically infected patients. One of the antigens preferentially recognized by IgG from the ocular samples is GRA2 (Klaren *et al*., 1998).

Definitive diagnosis of a *T. gondii* infection relies on isolation of the parasite. The research of the parasite or DNA can be made in different kinds of samples: amniotic fluid (Romand *et al*, 2001), foetal blood, placenta (Ajzenberg *et al*, 2002b), cord blood, blood (Bou *et al*, 1999; Chai *et al*, 2003; Hofflin and Remington, 1985; Joseph *et al*, 2002), cerebrospinal fluid (Joseph *et al*, 2002), cerebral biopsy, bronchoalveolar lavage (Gadea *et al*, 1995), aqueous humor (Bou *et al*, 1999), saliva and tonsils (Amendoeira and Coutinho, 1982).

Parasite isolation by mouse inoculation (bioassay) is possible from almost all kind of samples (biological fluid or digested tissue). The great disadvantage is that it takes about 6 weeks to have the results (by serology). Cell culture allows a rapid development of parasites. The results are obtained in a shorter period of time, but with a lower sensitivity than mouse inoculation, especially for the diagnosis of symptomatic congenital infections (Robert-Gangneux, 2001).

The search of *T. gondii* DNA is performed by polymerase chain reaction (PCR). Prenatal diagnosis of congenital toxoplasmosis using PCR in amniotic fluid was initially suggested by Grover *et al.* (1990). Several loci can be searched, being the B1 the most commonly used for the diagnosis (Bou *et al.*, 1999; Joseph *et al.*, 2002; Kompalic-Cristo *et al*., 2007; Romand *et al.*, 2001). Prenatal diagnosis using PCR on amniotic fluid was estimated to have a sensitivity of 64% and a specificity of 100% (Romand *et al*, 2001). In the diagnosis of TE, PCR can be used with high specificity and relatively high sensitivity on cerebrospinal fluid or blood samples (Joseph *et al.*, 2002). Monitoring for *Toxoplasma* DNA

in peripheral blood samples using PCR was indicated as a valuable method for identifying *Toxoplasma*-seropositive stem cell transplant recipients (Edvinsson *et al*., 2008). Molecular diagnosis can also be used to the diagnosis of ocular toxoplasmosis in aqueous humor samples (Bou *et al.*, 1999) as well as lens aspirates and peripheral blood leucocytes (Mahalakshmi *et al*., 2007).

In summary, a definitive diagnosis of *Toxoplasma* infection is based on isolation of the parasite, which is particularly important for the diagnosis of congenital infections and severe toxoplasmosis in immunocompromised patients. Since detection of parasite is not always possible, diagnosis based on serological methods is an essential diagnostic tool. The future of these serological methods will pass be using specific antigens as markers of infection.

1.5 *Toxoplasma gondii* antigens

Polymorphic antigens of *T. gondii* have been used to discriminate *Toxoplasma* strains. Four main classes of antigens will be here described with special emphasies to GRA proteins. These proteins (GRA) were used in the present work to define strain-specific peptides capable of discriminate *T. gondii* strains.

1.5.1 SAG proteins

Surface antigens called SRS (SAG1-related sequence) are membrane proteins. This superfamily comprises at least 20 homologous proteins and SAG1 is the prototypic member (Jung *et al*., 2004). These proteins play an important role in host cell adhesion and invasion (Grimwood and Smith, 1992; Mineo *et al*., 1993; Robinson *et al*., 2004), by acting in the initial recognition of the target cell. Most of these proteins belong to the SAG1 family (Lekutis *et al*, 2001). Proteins belonging to the SRS superfamily may also be involved in tachyzoite to bradyzoite switch, since a differential expression of these proteins is observed on both stages (Kim *et al*., 2007). *SAG* genes have been used for molecular characterization of *Toxoplasma* strains by PCR followed by restriction fragment length polymorphism (RFLP) or sequencing, specially *SAG1* (Dubey *et al*., 2006a; Howe and Sibley, 1995; Lin *et al*., 2005; Sibley and Boothroyd, 1992), *SAG2* (Howe *et al*., 1997; Lehmann *et al*., 2000; Su *et al*., 2006), *SAG3* (Grigg *et al*., 2001), and *SAG5C* (Tinti *et al*., 2003).

1.5.2 MIC proteins

Micronemes are secretory organelles in the tachyzoite and they discharge their contents from the apical end of the parasite. Microneme proteins (MIC) are the first proteins to be released, upon contact with the host cell and are thought to function in host cell recognition, attachment and penetration (reviewed by Carey *et al*., 2000; Sibley *et al*., 1998). MICs link the parasite actin–myosin system to the host surface, facilitating parasite entry into the host cell (reviewed by Hager and Carruthers, 2008). After discharge, some micronemal proteins remain associated with the parasite's apical surface where they are thought to

mediate attachment by binding to specific receptors on the target host cell (Carruthers and Sibley, 1997; Garcia- Réguet *et al*., 2000). MIC proteins may be presented as soluble proteins or as transmembrane proteins.

1.5.3 ROP proteins

Rhoptries are secretory/excretory organelles, exclusive of the Apicomplexa that are involved in the invasion of host cells and formation of the PV (reviewed by Dubremetz, 2007; El Hajj *et al*., 2006; reviewed by Sinai, 2007). So far, more than 30 rhoptry proteins were identified. There are two denominations for rhoptry proteins: ROP for those located in the posterior part and RON for those located in the neck of the organelle (reviewed by Dubremetz 2007). To date, all proteins secreted from the rhoptry either associate with the PVM or are transported across it into the host cell cytoplasm. The rhoptries discharge their contents from the apical end of the tachyzoite (reviewed by Carey *et al*., 2000).

Rhoptry proteins are synthesised as pre-pro-proteins that are processed first to pro-proteins upon entrance into the secretory pathway, and then processed again to their mature forms late in the secretory pathway (Bradley and Boothroyd, 2001). Some proteins are phosphorylated (ROP4, ROP9) (Carey *et al*., 2004; Reichmann *et al*., 2002). ROP2 and ROP4 are involved in host lactoferrin acquisition (Dziadek *et al*., 2007). ROP16 and ROP18 may be involved in parasite virulence (El Hajj *et al*., 2007; Saeij *et al*., 2006; Saeij *et al*., 2007; Taylor *et al*., 2006).

1.5.4 GRA proteins

Dense granules are secretory organelles of Apicomplexa parasites. These organelles are involved in the maturation of the PV where the parasite multiplies. They have approximately 200nm in diameter, surrounded by a unique membrane and are found during invasive stages of parasite. Dense granule (GRA) proteins occur as both soluble and aggregated forms and are mainly predicted to be Type I transmembrane proteins. However, they are only secreted as soluble proteins (reviewed by Mercier *et al*., 2005). GRA proteins form oligomeric complexes with their hydrophobic domains within the interior of the complex. This event may explain their solubility within the dense granules and the vacuolar matrix. The weight of these complexes seems to be influenced by the nature of the

hydrophobic domains. Transmembrane domains (GRA3, GRA5, GRA6, GRA7) form larger complexes, while amphipathic alpha-helices (GRA2, GRA9), smaller ones (Braun *et al*., 2008). During and after cellular invasion, the GRA proteins are secreted from tachyzoite dense granules into the PV. After release, the GRA proteins remain soluble in the lumen of the vacuole or become associated with the PVM (GRA3, 5, 7, 8) or the tubuloreticular network of membranes within the PV (GRA2, 4, 6, 9, 12) (Adjogble *et al*., 2004; reviewed by Mercier *et al*., 2005; Michelin *et al*., 2009). These proteins are involved in intracellular survival and replication of *T. gondii* (reviewed by Carey *et al*., 2000) by modifying the vacuolar membrane and possibly acquiring nutrients from the host cell (Cesbron-Delauw *et al*., 1996). GRA proteins are expressed by the three stages of *T. gondii*, tachyzoite, bradyzoite (Lecordier *et al*., 1995) and sporozoite (Tilley *et al*., 1997). GRA proteins size varies between 21-49 kDa. At the N-terminal they possess a hydrophobic sequence with characteristics of a signal peptide. The predicted site of cleavage occurs most of the time in the range from 19 to 27 amino acids. Release of GRA proteins into the vacuole can occur by a regulated pathway, which takes place shortly after formation of the PV (Carruthers and Sibley, 1997) or a constitutive, calcium-independent mechanism (Karsten *et al*., 1998). The constitutive release is enhanced by the ADP-ribosylation factor-1 (Liendo *et al*., 2001). In the dense granules of *Toxoplasma* tachyzoites, 12 GRA proteins (GRA1-GRA10, GRA12 and GRA14), 2 isoforms of nucleotide triphosphate hydrolase (NTPase I and II) and 2 protease inhibitors (TgPI 1 and 2) were identified (reviewed by Ahn *et al*., 2006; Michelin *et al*., 2009; Rome *et al*., 2008). Some GRA proteins are glycosylated: GRA2 (Zinecker *et al*., 1998), GRA4 (Achbarou et al., 1991), GRA6 (Mercier *et al*., 2005) and GRA12 (Michelin *et al*., 2009). GRA3, GRA4, GRA5, GRA6, GRA7, GRA8, GRA10 and GRA12 have transmembrane domains. GRA3 and GRA5 are associated with the PVM. GRA1, GRA2, GRA4, GRA6, GRA12, and NTPase are found within the intravacuolar space and are associated with the tubulovesicular network (reviewed by Mercier *et al*., 2005; Michelin *et al*., 2009). GRA9 has an amphipathic alpha helix (reviewed by Mercier *et al*., 2005) and GRA2 has three (Travier *et al*., 2008). Some GRA proteins (GRA3, GRA5, GRA6) are involved in modulation of intracellular calcium concentrations, through bound to the calcium modulating ligand (CAMLG), which leads to the inhibition of host cell apoptosis for the long-term residence of the intracellular parasites (Ahn *et al*., 2006).

In the present work, synthetic peptides used for serotyping assays were derived from GRA proteins. Peptides derived from GRA5, GRA6 and GRA7 proteins were previously described for serotyping by other authors with promising results (Kong *et al*., 2003; Peyron *et al*., 2006). Peptides derived from GRA8 were not described so far. Although, the degree of strain-specific polymorphisms of GRA8 is unknown, this antigen has one characteristic essential for serotyping: immunogenicity. Based on this, GRA8 was included in this study with the intent of define strain-specific peptides derived from this antigen.

1.5.4.1 GRA5

GRA5 is a 21 kDa protein, which is inserted into the PVM. This protein is released into the vacuole as a soluble form and then inserted into the vacuolar membrane. GRA5 is inserted into the PVM as a transmembrane protein with the N-terminal into the host cytosol and the C-terminal in the lumen of the vacuole (Lecordier *et al*., 1999). GRA5 has a hydrophobic domain (aa 76-93) that could form a membrane-spanning α helix (Lecordier *et al*., 1993). This domain is necessary for interaction of the protein within hydrophobic aggregates in dense granules and for insertion into the vacuolar membrane after secretion into the PV (Lecordier *et al*., 1999). Soluble trafficking of the PV-targeted GRA5 transmembrane protein is parasite specific. GRA5 is targeted to the plasma membrane and behaves as an integral membrane protein with a type I topology when expressed in mammalian cells. GRA5 N-terminal ectodomain prevents membrane integration within the parasite and is essential for both sorting and post-secretory membrane insertion into the vacuolar membrane (Gendrin *et al*., 2008).

1.5.4.2 GRA6

Lecordier and colleagues (1995) described for the first time a dense granule antigen of 32 kDa, which was named GRA6. In extra cellular parasites, GRA6 exists in dense secretory granules mostly as soluble proteins. Like the other GRA proteins, GRA6 is involved in host cell invasion. GRA6 is a glycine-rich protein, and behaves as an integral membrane protein within the parasitophorous vacuole (Labruyere *et al*., 1999). *GRA6* locus is a single copy gene, does not contain introns (Lecordier *et al*., 1995) and is localized at chromosome X (Khan *et al*., 2005b). It potentially encodes a 230-amino-acid polypeptide that contains two hydrophobic regions with the characteristics of transmembrane domains. The first

hydrophobic domain is N-terminally located. The second domain is central and is flanked by two hydrophilic domains (Lecordier *et al*., 1995). GRA6 stabilizes the tubules forming the network within the vacuolar space (Mercier *et al*., 2002). Throughout the development of the vacuole within the host cell, GRA2 and GRA6 remain associated with the network membranes in a stable multimeric complex that also includes GRA4 (Labruyere *et al*., 1999).

GRA6 is a highly polymorphic protein and this characteristic has been explored for the molecular characterization of *Toxoplasma* strains (Belfort-Neto *et al*., 2007; de Sousa *et al*., 2008; Dubey *et al*., 2006a; Dubey *et al*., 2006b; Dubey *et al*., 2006c; Dubey *et al*., 2006d; Dubey *et al*., 2007b; Dubey *et al*., 2007c; Dubey *et al*., 2007d; Dubey *et al*., 2007e; Dubey *et al*., 2007f; Dubey *et al*., 2007g; Dubey *et al*., 2008a; Dubey *et al*., 2008b; Fazaeli *et al*., 2000; Khan *et al*., 2005a; Khan *et al*., 2006; Lin *et al*., 2005; Miller *et al*., 2004; Petersen *et al*., 2006). Sequencing of *GRA6* detected a high polymorphism (Fazaeli *et al*., 2000; Zakimi *et al*., 2006b). Single nucleotide polymorphisms (SNP) in *GRA6* coding region can be detected by methods based on PCR-RFLP: digestion of amplification products with a single endonuclease (MseI) can differentiate genotypes I, II, and III (Fazaeli *et al*., 2000). Another method based on *GRA6* polymorphisms is pyrosequencing (Edvinsson *et al*., 2007). This technique allows analysing short DNA sequences and SNP. Two SNP located at positions 162 and 171 of the *GRA6* gene allow differentiating type I, II and III. GRA6 was also proposed for serotyping. Peptides selected from the polymorphic C-terminal region were used to differentiate type II from type I and III (Kong *et al*., 2003; Peyron *et al*., 2006).

1.5.4.3 GRA7

GRA7 is a dense granule antigen of 29 kDa (Fisher *et al*., 1998; Jacobs *et al*., 1998). It is an intron-free gene (Fisher *et al*., 1998). The deduced 236 amino acid protein contains a putative N-terminal signal peptide (aa 1-26), one site of potential N-linked glycosylation and, close to the C-terminus, a further hydrophobic, putative transmembrane domain (Fisher *et al*., 1998; Jacobs *et al*., 1998). In tachyzoite-infected cells, GRA7 is accumulated within the parasitophorous vacuole and is co-localized with its delimiting membrane. In bradyzoite-infected cells, GRA7 is present within the host cell cytoplasm (Fisher *et al*., 1998). This protein shares from aa 1 to 33 and from 108 to 212, 47% identity with a *Neospora caninum* GRA (Fisher *et al*., 1998). Three possible signal peptidase cleavage sites are found (positions

18, 21 and 26, respectively). The signal sequence contains the only cysteine of the protein. There are no tryptophan residues present and the proline content is 5.5% (Jacobs *et al*., 1998).

Like GRA6, it is involved in host cell invasion. This protein is associated with the parasite membrane complex, with the tubular elements of the intravacuolar network and with the PVM. It migrates from the dense granules to the PVM through the intravacuolar network during host cell invasion (Bonhomme *et al*., 1998). GRA7 is involved in sequestring host endo-lysosomes within the PV (Coppens *et al*., 2006).

1.5.4.4 GRA8

GRA8 is an intron-free gene with an open reading frame of 801 bp (Carey *et al*., 2000). The deduced aa sequence of GRA8 (38 kDa) consists of a polypeptide of 267 aa. The sequence contains an amino terminal signal peptide (a hydrophobic region of 23 amino acids), three degenerate proline-rich repeats in the central region and a potential transmembrane domain near the carboxy terminal region (a second hydrophobic region spanning aa 223 to 242). The predicted cleavage site is located between alanine 23 and methionine 24 (Carey *et al*., 2000). GRA8 is released into the PV during or shortly after invasion and associates with the periphery of the vacuole.

1.5.5 Serological response in humans against recombinant antigens

Most serological tests for *Toxoplasma* require the preparation of parasite antigens (whole extract) from tachyzoites harvested from mice or cell culture systems. Tachyzoites and whole extract antigen production is a laborious and expensive task that may vary between laboratories. Besides, methods based on whole extract antigens are difficult to standardise, since antigen may contain material from the host cells. Therefore, recent advances have been made in generating recombinant antigens of *T. gondii* which are less expensive and easier to standardize in IgG and IgM serological tests.

The use of recombinant *T. gondii* antigens in the diagnostic has been evaluated. Recombinant antigens seem to be also useful for differentiating recently acquired infection from those acquired in the more distant past (Table 2).

Table 2: ELISA sensitivity of some recombinant proteins against serum samples from acute and chronic infections.

Recombinant protein	Sensitivity		Reference
	Acute	Chronic	
GRA1	34%	78.2%	Ferrandiz *et al*., 2004
GRA1	83.3%	80.5%	Pietkiewicz *et al*., 2004
GRA2	95.8 to 100%	65.7 to 71.4%	Golkar *et al*., 2007
GRA2	82.6%	75%	Murray *et al*., 1993
GRA6	89%	—	Redlich and Muller, 1998
GRA7	94%	65 to 79%	Jacobs *et al*., 1999
GRA7	68.9%	78%	Pietkiewicz *et al*., 2004
GRA8	85.3%	8%	Li *et al*., 2000b
GRA8	90%	0%	Suzuki *et al*., 2000
GRA8, P22, MIC5 and GRA7	90%	1.4%	Li *et al*., 2000a
SAG1	98.6%	90.2%	Pietkiewicz *et al*., 2004
SAG2	80%	100%	Fong *et al*., 2008
GRA1, GRA7 and SAG1	100%	97.6%	Pietkiewicz *et al*., 2004

Many GRA proteins are immunogenic: GRA1 (Beghetto *et al*., 2003), GRA2 (Murray *et al*., 1993), GRA3 (Beghetto *et al*., 2003), GRA4 (Chardès *et al*., 1993), GRA6 (Lecordier *et al*., 2000), GRA7 (Beghetto *et al*., 2003), GRA8 (Beghetto *et al*., 2003). This property has been used in the development of serodiagnosis methods using recombinant GRA proteins.

In the study performed by Lecordier and coleagues (2000), a recombinant GRA1 protein was shown to have a low sensitivity (68%) for IgG ELISA. GRA1 was described as a good marker of chronic phase (Cesbron-Delauw *et al*., 1989; Ferrandiz *et al*., 2004). GRA1 was show to be more sensitive in chronic infections (78.2%) than in acute infections (34%) (Ferrandiz *et al*., 2004). However, in the study of Pietkiewicz and colleagues (2004) sensitivity of rGRA1 was similar for acute (83.3%) and chronic infections (80.5%).

GRA2 is a highly immunogenic protein that can be used as a marker for the serodiagnosis of acute infection (Golkar *et al*., 2007). An ELISA using a GRA2 recombinant protein showed a sensitivity ranging from 95.8% (France) to 100% (Iran) in serum samples from pregnant women with acute infection. For serum samples from chronic infections the sensitivity ranged from 65.7% (France) to 71.4% (Iran) (Golkar *et al*., 2007). The 50 C-terminal residues of GRA2 expressed in fusion with glutathione-S-transferase (GST) when used in an IgG-ELISA reached 75% sensitivity for chronic infections and 82.6% for acute infections (Murray *et al*., 1993).

Two recombinant GRA6 proteins were studied by Lecordier *et al* (2000). The recombinant protein with the GRA6 C-terminal showed a sensitivity of only 10% by ELISA, while the GRA6 N-terminal fusion protein showed a sensitivity of 96% (Lecordier *et al*, 2000). GRA6 is considered a good marker of acute infection (Gatkowska *et al*., 2006; Redlich and Muller, 1998). An IgG-ELISA using a recombinant GRA6 antigen had a sensitivity of 89% for acute infections (Redlich and Muller, 1998). IgG and IgM ELISAs with rGRA6 are useful to identify and discriminate recent from past *Toxoplasma* infection in pregnant women. To discriminate acute from chronic infections, the GRA6–IgG-ELISA reached sensitivity and specificity of 87.5% and 94.1%, respectively. GRA6–IgM-ELISA reached a sensitivity and specificity of 91.7% and 97.1%, respectively, for detection of recently acquired infection. The GRA6–IgM-ELISA had a meaningful correlation with Vidas Toxo IgM and exhibited higher specificity (97.1%) than Euroimmun IgM ELISA (88.2%) (Euroimmun, Lübeck, Germany) for detection of recent infection (Golkar *et al*., 2008). However, in other study using a different recombinant antigen GRA6 N-terminal presents an overall sensitivity of 83%, with no significant difference between acute and chronic infection in pregnant women. This recombinant antigen can not be used to differentiate between acute and chronic toxoplasmosis (Ferrandiz *et al*, 2004).

GRA7 is an antigen characteristic of the acute phase of the infection (Gatkowska *et al.*, 2006; Pfrepper *et al*., 2005; Pietkiewicz *et al*., 2004) and a target antigen in intracerebral immune response during chronic phase of infection (Fatoohi *et al*., 2002; Neudeck *et al*., 2002). An IgG ELISA based on the detection of GRA7 expressed in *Escherichia coli* as a polyhistidine fusion protein has been reported to reach 81% sensitivity (Jacobs *et al*. 1999). However, when serum samples from acute and chronic infections were analysed separately, different sensitivity was found for the two groups. For acute infections, sensitivity was 94%, while for chronic infections, sensitivity was only 79%. Chronic infections with low titers were even more difficult to detect, reaching a sensitivity of 65% (Jacobs *et al*., 1999). Another study using rGRA7 showed a sensitivity of 68.9% for acute infections and 78% for chronic infections (Pietkiewicz *et al*., 2004). GRA7 and GRA4 antigens were shown to be capable of differentiate congenitally infected children from non-infected especially in patients younger than 4 months (Altcheh *et al*., 2006). The recombinant GRA7 and GRA4 antigens had a sensitivity of 64% and 100% respectively in children with less than 4 months, while for older children sensitivity was 11% and 30% respectively.

GRA8 is also an immunogenic protein (Beghetto *et al*., 2003). Antibodies against P35 (GRA8) are produced during acute phase of infection. An IgG-ELISA using a P35 recombinant antigen recognized 85.3% of the acute infections and only 8% of the chronic infections (Li *et al*., 2000b). An other study, also with a recombinant P35 antigen showed by IgM-ELISA a sensitivity of 90% in serum samples from pregnant women with recent infections and a 0% sensitivity in chronic infections. Serum samples with persistent IgM had a sensitivity of 25% (Suzuki *et al*., 2000). However, another study showed that IgG antibodies against GRA8 persist far beyond the acute phase of infection, which limits the use of this antigen as a marker of acute infection (Pfrepper *et al*., 2005). P35 recombinant antigen combined in an ELISA with P22, P25 (MIC5) and P29 (GRA7) recombinant antigens was able to detect 90% of the acute infections. However, these combined ELISA did not detect 98.6% of the chronic infections (Li *et al*., 2000a). The results reported by Aubert and colleagues (2000) suggest that the combination of P29, P30, and P35 in a IgG rec-Elisa and the combination of P29, P35, and P66 in a IgM rec-ELISA, can replace the tachyzoite antigen in IgG and IgM serologic tests, respectively. The IgG rec-ELISA had a sensitivity of 98.4% and the IgM rec-ELISA had a sensitivity of 79.2%.

Some SAG proteins such as SAG1 (Aubert *et al*., 2000; Decoster *et al*. 1988; Gatkowska *et al*., 2006; Gross *et al*. 1992; Pfrepper *et al*., 2005; Santoro *et al*. 1985; Velmurugan *et al*., 2008) and SAG2 (Lau and Fong, 2008; Parmley *et al*., 1992) are immunogenic and that property has been tested for serological diagnosis.

Recombinant antigen SAG1 was tested against serum samples from patients with acute and chronic infections. Sensitivity of recombinant antigen for acute infections was 98.6% while for chronic infections was 90.2% (Pietkiewicz *et al*., 2004). A recombinant protein from SAG2 antigen was recognized by all *Toxoplasma* positive serum samples by western blot. However, ELISA sensitivity varied from 80% in patients with acute infection to 100% in patients with chronic infections (Fong *et al*., 2008).

An IgG-ELISA using a C-terminal ROP2 recombinant antigen had a sensitivity of 89% (van Gelder *et al*., 1993). Three recombinant proteins derived from NTPase had a sensitivity that varies from 63% to 82% against human serum samples by ELISA (Nakajima-Nakano *et al*., 2000).

MIC1, MIC3 and MIC5 are strongly immunogenic and may represent a promising tool for human serodiagnosis (Beghetto *et al*., 2003; Holec *et al*., 2008). *T. gondii* proteins MIC2, MIC3, M2AP, GRA3, GRA7 and SAG1 were used to construct two recombinant chimeric antigens. Chimeric antigen GST-EC2 was constructed with the antigens MIC2, MIC3 and SAG1, while GST-EC3 was constructed with the antigens GRA3, GRA7 and M2AP. These two recombinant proteins reacted with 100% of IgG positive sera by ELISA. Reactivity of serum samples IgM positive from adults and children from mother with a primary infection during pregnancy was different. In adults, 98% of the sera reacted with the recombinant antigen GST-EC2, while only 84% reacted with the antigen GST-EC3. Reactivity of children was lower for both antigens. Antigen GST-EC2 was recognized by 70%, while GST-EC3 was recognized by 50% of the children (Beghetto *et al*., 2006). Although, only 70% of the children with congenital infection were positive for these recombinant antigens, this percentage falls to 35% with commercial kits.

Although, individual reactivity against the different recombinant antigens is not always especially high, the combination of more than one recombinant antigen increases the sensitivity of the ELISA. Individual sensitivity of recombinant GRA1 and recombinant GRA6

was 68% and 96% respectively, while the study of these two antigens increases the ELISA sensitivity to 98% (Lecordier *et al*., 2000). Sensitivity of an IgG-ELISA with three recombinant antigens from GRA1, GRA7 and SAG1 was 97.6% for chronic infections and 100% for acute infections. The individual sensitivity of each of these antigens for the same sera was lower, as already referred (Pietkiewicz *et al*., 2004). A combination of SAG1, GRA1 and GRA7 recombinant antigens may replace the native lysed, whole-cell antigens in the avidity test for IgG antibodies against *T. gondii* (Pietkiewicz *et al*., 2007).

In summary, specific antigens of *T. gondii* are involved in invasion of host cells and formation of PV. Many of these antigens have immunogenic characteristics that make them good candidates for serodiagnosis. However, the immune response against some of these antigens is very heterogenic. *Toxoplasma* antigens are also polymorphic. This characteristic has been used for molecular characterization of *T. gondii* strains.

1.6 Genotyping of *Toxoplasma gondii* isolates

1.6.1 Technical tools

From a technical point of view, many tools usable for genetic studies have been used: RFLP (Sibley and Boothroyd, 1992), PCR-RFLP (Binas and Johnson, 1998; Howe and Sibley, 1995; Howe *et al*., 1997; Sibley and Boothroyd, 1992), sequencing (Ajzenberg *et al*., 2004; Fazaeli *et al*., 2000; Høgdall *et al*., 2000; Lehmann *et al*., 2000), isoenzyme analysis (Dardé *et al*., 1992) and length polymorphism of microsatellite sequences (Ajzenberg *et al*., 2002a; Ajzenberg *et al*., 2005; Costa *et al*., 1997).

1.6.1.1 Isoenzymes and RFLP

Isoenzymes were the first analytical tools to be used in an attempt to biochemically characterize *Toxoplasma,* when *Toxoplasma* genotypes were not known (Dardé *et al*., 1988). Zymodemes were defined based on the isoenzymatic profile and an attempt was made to establish a relationship between isoenzymatic profiles and strain pathogenicity in mice (Dardé *et al*., 1992).

One mutation at the DNA sequence may result in a new codon, which can be translated as a different aa. If the new aa has a different electric charge, the protein will have a different migration pattern. The polymorphisms detected by RFLP or isoenzymatic analysis are related with the detection of one mutation in the DNA sequence at gene level (RFLP) or at gene function (isoenzymes).

The major problem of these two techniques is that by RFLP only the mutations for the restriction sites are detected, and by isoenzymatic analysis, information is lost when the mutation is silent (another codon, but the same aa is translated).

1.6.1.2 PCR-RFLP

PCR-RFLP on single-copy genes is the most commonly used method for typing *T. gondii* isolates.

DNA nucleotide polymorphism can change the restriction site for a restriction enzyme. The local for a restriction enzyme can be present in some strains and absent in others. If the restriction site is absent, the enzyme does not cut the DNA. If the restriction site is present, the enzyme will cut the DNA in two fragments. The length of those fragments will be different according with strain. This method is rapid and easy to use, but technical problems, such as incomplete amplification or incomplete digestion of the DNA by restriction enzymes have been reported.

A monolocus *SAG2* PCR-RFLP method was one of the most commonly used technique. In 5' end exists a restriction site for the enzyme Sau3AI, and in the 3' end a restriction site for the enzyme HhaI. The strains with no restriction site for these two enzymes are the allele 1 of *SAG2* gene. The strains with the restriction site for Sau3AI are allele 3 of *SAG2*. The strains with the restriction site for HhaI are allele 2 of *SAG2* (Howe *et al*., 1997).

But a valuable genotyping must be multilocus typing. A multilocus RFLP based on the following markers was developed: *SAG1, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1*, a new *SAG2* and *Apico* (Dubey *et al*., 2006a; Dubey *et al*., 2007e; Su *et al*., 2006). Multilocus RFLP has a higher discriminative power and allow the differentiation of non-archetypal genotypes. A new *SAG2* marker was developed by Su *et al* (2006). A single fragment of 546bp is amplified and can differentiate all three alleles in one double-digestion reaction. This new marker contains SNPs recognized by the restriction enzyme *Hinf I* in the *SAG2*-5' and by the *Taq I* in the upstream coding region of *SAG2*. Enzyme *Hinf I* cuts the allele I and III sequences. *Taq I* cuts type II and some atypical alleles. Marker *SAG3* required only *Nci I* to discriminate the three archetypal alleles (Grigg *et al*., 2001). Markers *c22-8, c29-2* and *PK1* are characterized by the three archetypal alleles and also by some non-archetypal alleles (Su *et al*., 2006). Digestion of marker *c22-8* with *BsmA I* and *Mbo II* and marker *c29-2* with *HpyCH4IV* and *Rsa I* revealed a fourth non-archetypal allele (u-1). Digestion of *PK1* with *Ava I* and *Rsa I* revealed a characteristic fourth (u-1) and fifth (u-2) alleles. Marker *PK1* was developed based on *T. gondii* protein serine/threonine kinase (PK1) gene sequence described by Ng *et al*. (1997). *Apico* marker for apicoplast genome, digested with *Afl II* and *Dde I,*

clearly distinguish the three archetypal lineages (I, II, III) (Su *et al*., 2006). The coding region of *GRA6* locus is a considerable polymorphic target. It can clearly differentiate the three archetypal genotypes (I, II and III) by using a single PCR reaction followed by a single endonuclease digestion with *MseI* (Fazaeli *et al*., 2000). In the study performed by Fazaeli and colleagues (2000) a high polymorphism was detected with *GRA6* sequence analysis, but the *GRA6* PCR-RFLP method which was developed could simply differentiate three different groups among the same strains, missing all atypical alleles. A double digestion of *BTUB* maker with *BsiE I* and *Taq I* distinguish the three archetypal alleles (Su *et al*., 2006). Differentiation of type I strains from type II and III using *SAG1* marker, was initially based on the digestion of the PCR product by the enzymes *DdeI*, *Sau96I* and *HaeII* (Sibley and Boothroyd, 1992). Virulent strains have restriction sites for *DdeI* and *Sau96I*. Non virulent strains have a restriction site for *HaeII*. Recently a double digestion of *SAG1* PCR products with *Sau 96I* and *Hea II* (Dubey *et al*., 2006a) allowed to differentiate a unique *SAG1* atypical allele (Dubey *et al*., 2006d; Dubey *et al*., 2007c; Dubey *et al*., 2007g; Sundar *et al*., 2008).

Other markers have also been described for RFLP. The *B1* gene is not sufficiently polymorphic to allow strain typing (Grigg and Boothroyd, 2001). Marker *B1* distinguishes type I from type II and III by RFLP. Type I strains are not digested by *Xho I* neither by *Pml I*, while type II and type III strains are digested by both enzymes at *B1* marker (Grigg and Boothroyd, 2001).

GRA7 has been less explored as a *Toxoplasma* genotyping marker. Preliminary results (Villena personal data, unpublished) showed that *GRA7* allows the discrimination between genotypes I, II, III and some atypical strains by PCR-RFLP.

Three other markers were described for RFLP: *cB21-4*, *L363* and *cS10-A6* (Ferreira *et al*., 2006; Su *et al*., 2002). Digestion of *cS10-A6* with *RsaI* and *HpyCH4IV* differentiate the three clonal types (I, II and III). Digestion of *L363* with *MspI* and *HpyCH4IV* reveals a fourth characteristic allele. Marker *L363* may be a possible marker of highly virulent strains, since in a study conducted with Brazilian strains, only strains belonging to group I-A (highly virulent) showed the haplotype I in this locus (Ferreira *et al*., 2006). Marker *cB21-4* is a highly polymorphic marker. Seven different alleles were found in Brazilian strains by digesting this marker with the enzyme *Hea III* (Ferreira *et al*., 2006).

1.6.1.3 Sequencing

Sequencing means identifying the sequence of nucleotides in DNA. By comparing the sequence of a gene from several strains, all mutations present in the gene are detected, and by doing so the real polymorphism of the gene. DNA sequencing can be used as a typing method to detect SNPs at various base pair level or deletions or as a method to detect polymorphic endonucleases restriction sites in order to develop a PCR-RFLP method. According to the technique, a same marker could reveal more or less polymorphism. In the study performed by Fazaeli and colleagues (2000) a high polymorphism was detected with *GRA6* sequence analysis, but the *GRA6* PCR-RFLP method which was developed could simply differentiate three different groups among the same strains. Sequencing is often used as complement of RFLP genotyping in order to define new alleles. Type X and type A defined for sea otters were shown to be genetically different from type II strains based on *B1, SAG1* and *GRA6* sequencing (Miller *et al*., 2004; Sundar *et al*., 2008). The *GRA6* RFLP polymorphism specific for type II was shared by type X strains. An isolate from an ocular infection from Korea was classified as type I by RFLP. However sequencing of *SAG1, ROP1* and *GRA8* loci revealed nucleotide polymorphisms and aa substitutions (Lin *et al*., 2005). Lehmann and colleagues (2000) performed a study, focused on sequence variation in introns of housekeeping genes, which may be more informative for phylogenetic analysis because they evolve under lower selection. They compared the genetic diversity in introns of 5 housekeeping genes, with coding and noncoding regions of 2 antigen-coding genes. Introns of housekeeping genes were more informative than coding and noncoding regions of antigen-coding genes, but the difference between the markers was rather small. Uracil phosphoribosyl transferase (*UPRT*) intron 1 sequence of type II and III are identical, while type I possess a unique haplotype distinguished by 6 single nucleotide polymorphisms (Khan *et al*., 2005a). A new allele in *Toxoplasma* strains from Brazil was described for the UPRT-1 intron that was distinguished of the clonal lineages by six polymorphisms (Khan *et al*., 2006).

1.6.1.4 Pyrosequencing

Pyrosequencing was first described by Ronaghi and colleagues (1998). It is being used for genotyping bacteria, viruses and metazoan parasites (reviewed by Sreekumar *et al*., 2005). This technique allows analysing short DNA sequences and SNPs. Pyrosequencing is a bioluminometric method, where an enzymatic cascade reaction is used to convert the

inorganic pyrophosphate released during the incorporation of deoxynucleotide triphosphate, into proportional amounts of visible light that can be measured. Pyrosequencing is a simple, rapid and efficient method for genotyping of *T. gondii* that allows the detection and quantification of mixed genotype infections. Two markers were described for this method: *SAG2* (Sreekumar *et al*., 2005) and *GRA6* (Edvinsson *et al.*, 2007).

1.6.1.5 Microsatellites

Microsatellites (MS) are short tandem repeats of two to six nucleotides. Markers generated from these repeats are highly polymorphic because of length variation of these repeats. Consequently, they exhibit multiple alleles, which make them very informative for genetic studies. Microsatellites are considered the most informative technical tools for the study of DNA polymorphism. Hypervariability of MS is explained by the accumulation of length mutations by intra-allelic polymerase slippage on microsatellite sequence during replication. Polymorphism can be evaluated by PCR, which requires only a small amount of DNA, and allele sizing can be achieved with fluorescent primers and an automatic sequencer which assures reliability of the results.

The high discriminatory power of MS appears to be very useful in epidemiological studies of *T. gondii*, for detection of mixed infections or for parasite identification. Since *T. gondii* is haploid, only one peak is expected for a given locus corresponding to one allele. More than one peak will be detected if mixed infections with different alleles are present in the sample.

As the rates of mutation of MS are much higher compared with that of point mutations in RFLP markers, MS are especially useful in revealing very recent mutations in closely related isolates within a lineage, whereas RFLP markers are the better choice for testing more distantly related strains into different clonal branches.

The microsatellite (TG) in the beta-Tubulin gene of *T. gondii* distinguishes the strains virulent in mice, from others, which are avirulent. The virulent strains had a (TG)8 microsatellite, and the avirulent strains had a (TG)7 microsatellite. This microsatellite marker alone is not sufficient to type the strains which are avirulent in mice (Costa *et al*., 1997).

Other MS markers have been described for *Toxoplasma* genotyping. A multiplex PCR assay for multilocus typing based on length polymorphism of five MS markers was developed (Ajzenberg *et al*., 2005). Five MS markers were chosen: *TUB2* $(CA)_n$, a new MS in the beta-Tubulin gene, *TgM-A* $(TG)_n$ in the Myosin A gene, *W35* $(TC)_n(TG)_n$ (Ajzenberg *et al*., 2002a; Ajzenberg *et al*., 2004; Ajzenberg *et al*., 2005), *B17* $(TC)_n$, and *B18* $(CA)_n$ (Ajzenberg *et al*., 2004; Ajzenberg *et al*., 2005). The combination of these MS markers will provide enough information to distinguish strains.

The markers referred in the text are summarized in Table 3.

Table 3: Principal markers for *Toxoplasma* genotyping

Marker	Chromosome	Reference
SAG1	VIII	Sibley and Boothroyd, 1992;Dubey *et al*., 2006a
SAG2	VIII	Howe *et al*., 1997; Lehmann *et al*., 2000 ; Su *et al*., 2006
SAG3	XII	Grigg *et al*., 2001
SAG4	VIIa	Grigg *et al*., 2001
GRA6	X	Fazaeli *et al*., 2000; Khan *et al*., 2005a, b ; Su *et al*., 2006
GRA7	VIIa	Villena personal data, unpublished
GRA8	III	Lin *et al*., 2005
ROP1	XI	Lin *et al*., 2005
B1	IX	Grigg and Boothroyd, 2001
TgM-A	X	Ajzenberg *et al*., 2002a; 2004; 2005
W35	II	Ajzenberg *et al*., 2002a, 2004, 2005
B17	XII	Ajzenberg *et al*., 2004, 2005
B18	VII	Ajzenberg *et al*., 2004, 2005
βTUB (TUB2)	IX	Costa *et al*., 1997; Ajzenberg *et al*., 2002a, 2004, 2005; Khan *et al*., 2005a, b ; Su *et al*., 2006
C22-8	Ib	Khan *et al*., 2005b and Su *et al*., 2006
C29-2	III	Khan *et al*., 2005b and Su *et al*., 2006
L358	V	Khan *et al*., 2005b and Su *et al*., 2006
L363	VIIb	Ferreira *et al*., 2006
PK1	VI	Khan *et al*., 2005b and Su *et al*., 2006
Apico	Plastid	Su *et al*., 2006

1.6.2 *Toxoplasma gondii* population structure

Population genetic analysis can only be performed on a large population of isolates. This population should be representative of *T. gondii* circulating in nature. Different geographic origins, with isolates originating from different parts of the world, are necessary to appreciate the real genetic diversity of *T. gondii.* A study with a large number of strains isolated from different geographical environments, revealed four distinct populations (Lehmann *et al*., 2006). One population is found in all continents except South America, two other populations are confined to South America and a fourth population with worldwide distribution. The two populations characteristic of South America and the high genetic diversity found in that continent evidence that *Toxoplasma* evolved longer in that region, which have led the authors to suggest that South America was the birthplace of *Toxoplasma*. On the contrary, the population with the widest geographical range possesses the lowest population divergence and the highest divergence from the ancestral population indicating that it has evolved most recently. Migratory events from South America to Eurasia and from Europe to North America and Southeast Asia may explain the actual *Toxoplasma* distribution (Lehmann *et al*., 2006).

Khan and coleagues (2007) defined 11 haplogroups among 46 *Toxoplasma* strains. The haplogroups had a distinct geographic distribution. Similarly to the results obtained by Lehmann and colleagues (2006), some haplogroups were almost exclusively found in South America (haplogroups 4, 5, 8 and 9) and one (haplogroup 6) is globally distributed. Based on the frequencies of SNPs in each population it was estimated that Northern and Southern parasite populations diverged from a common ancestor in isolation over a period of ~10^6 yr. The initial divergence between North and South American population of *T. gondii* coincides with the reconnection of the Panamanian land bridge, which facilitated the southerly migration of definitive hosts and of *T. gondii*. These events may have led to the divergence of southern strains from pre-existing Northern ones. The three clonal lineages and some clonal South America haplogroups (4, 6b, 8 and 9) share a monomorphic version of chromosome Chr1a. This chromosome has emerged and spread within the past 10,000 years.

1.6.2.1 Archetypal clonal lineages

The main criteria for a clonal population structure in *T. gondii* are: the isolation of identical multilocus genotypes over large geographic areas and at interval of several years, the small number of different multilocus genotypes and the over-representation of the observed genotypes by comparison with panmixic expectations providing evidence of a high linkage disequilibrium, and the correlation between independent set of markers.

Multilocus typing is necessary to appreciate the real genetic diversity of *T. gondii* population, to find genetic factors that could influence virulence, to understand an eventual mechanism of genotype selection according to host species or to try to find relationships between human disease and genotype.

Studies based essentially on multilocus restriction fragment polymorphism analysis or on multilocus enzyme electrophoresis, indicated that *T. gondii* comprises three clonal lineages (Howe and Sibley, 1995). These studies showed a weak polymorphism of the markers.

More recently, a multilocus study with Brazilian strains suggested that although these strains were more closely related to the type I lineage, two groups could be defined based on mice virulence: I-A and I-B. Group I-A comprises strains highly virulent and were clustered with RH strain. Group I-B (cystogenic strains) strains had a heterogeneous behaviour with some virulent strains in mice (Ferreira *et al.*, 2006).

There are several explanations for the existence of clonal population structure in *T. gondii*. First, this parasite is able to transmit among intermediate hosts through carnivorism and scavenging, bypassing sexual recombination events in definitive host cats (Howe and Sibley, 1995; Su *et al.*, 2003). Second, many macrogametes of the parasite remain unfertilised but are capable of forming oocysts in the small intestine of cats by parthenogenesis (Ferguson, 2002). Third, cats simultaneously infected with different strains of *T. gondii* are likely to be very rare events in nature; therefore there is a temporal barrier for recombination to occur.

Sexual crosses between the ancestral lineages seem to be at the origin of the three clonal archetypal lineages (type I, II and III). Boyle and colleagues (2006) suggested that two separate crosses between ancestral versions of the present day type II strain and two distinct

strains may be at the origin of type I and type III. The type II parental strain is divergent from the other parental strains (called α and β by the authors), which are themselves distinct but more closely related to one another than they are to type II. A cross between a type II strain (type II1) and strain α resulted in the creation of the type I clonotype. A cross between type II3 and strain β produced the type III clonotype (Figure 5). Types I and III are second- and first-generation offspring, respectively, of a cross between a type II strain and one of two ancestral strains.

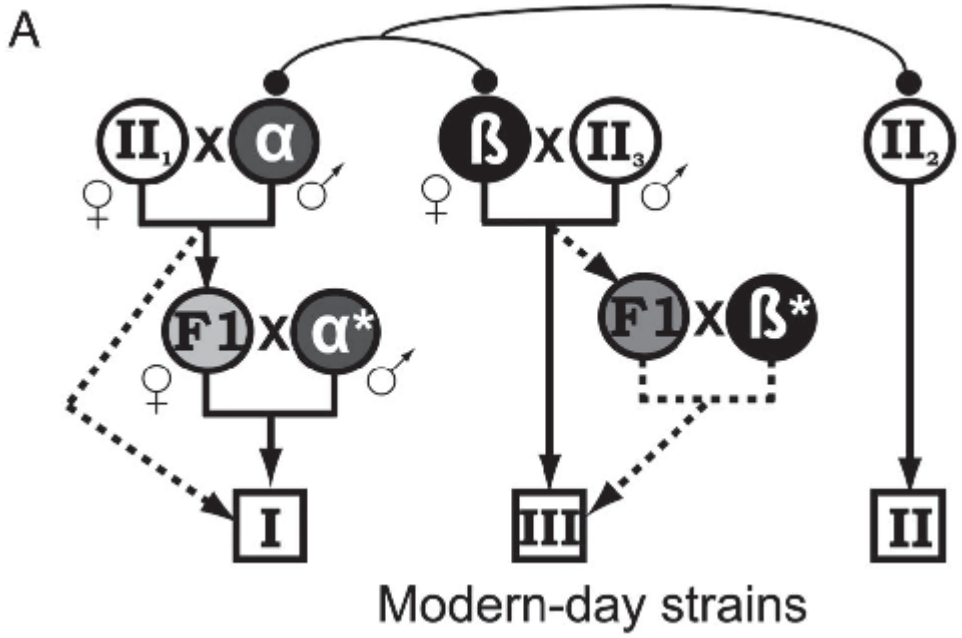


Figure 5: Proposed genealogy of three major *T. gondii* lineages and chromosome segregation during the proposed crosses (From Boyle et al., 2006).

1.6.2.2 Non-archetypal strains

Ajzenberg and colleagues (2004) performed a study with multi-locus sequencing of five microsatellite markers for 43 *T. gondii* strains from different geographical and host origins to reconsider the population structure and develop a more realistic picture of diversity between *Toxoplasma* strains by phylogenetic and molecular analysis. According to this study, while most strains fall into the three clonal lineages, the strains from French Guiana were

clearly atypical. Each French Guiana strain has a unique multi-locus genotype, and some of its alleles are not observed in strains from other areas. In this work, the author hypothesized that *T. gondii* presents a complex population structure with a mix of clonal and sexual propagation.

Clonal propagation also characterizes some South American strains (Khan *et al*., 2007; Pena *et al*., 2008). Four clonal lineages were recently defined for the Brazilian strains, designated as Types BrI, BrII, BrIII and BrIV (Pena *et al*., 2008). Type BrI is highly virulent, type BrIII is non-virulent, whilst Type BrII and BrIV are intermediately virulent in mice (Table 4).

Table 4: Comparison between genetic profile of type I, II and III clonal lineages and the Brazilian clonal lineages BrI, BrII, BrIII and BrIV (Pena et al., 2008).

Strain type	**PCR-RFLP genotype**											
	SAG1	***SAG2***	***SAG2* new**	***SAG3***	***BTUB***	***GRA6***	***C22–8***	***C29–2***	***L358***	***PK1***	***Apico***	***CS3***
I	I	I	I	I	I	I	I	I	I	I	I	-
II	II/III	II	II	II	II	II	II	II	II	II	II	-
III	II/III	III	III	III	III	III	III	III	III	III	III	-
BrI	I	I	I	III	I	II	u-1	I	I	I	I	I
BrII	I	I	II	III	III	III	I	III	I	II	III	I
BrIII	I	III	III	III	III	III	II	III	III	III	III	III
BrIV	u-1	I	II	III	III	III	u-1	I	I	III	I	-

The phylogenetic network of isolates from Brazil is highly reticulated, suggesting high recombination rates in the parasite population. The clonal type I and several genotypes from Brazil are clustered together. The clonal type III, type BrIII and several Brazil genotypes are

clustered into a major branch (Figure 6). The network highlights the phylogenetic distance of Brazilian strains from the archetypal type II strains.

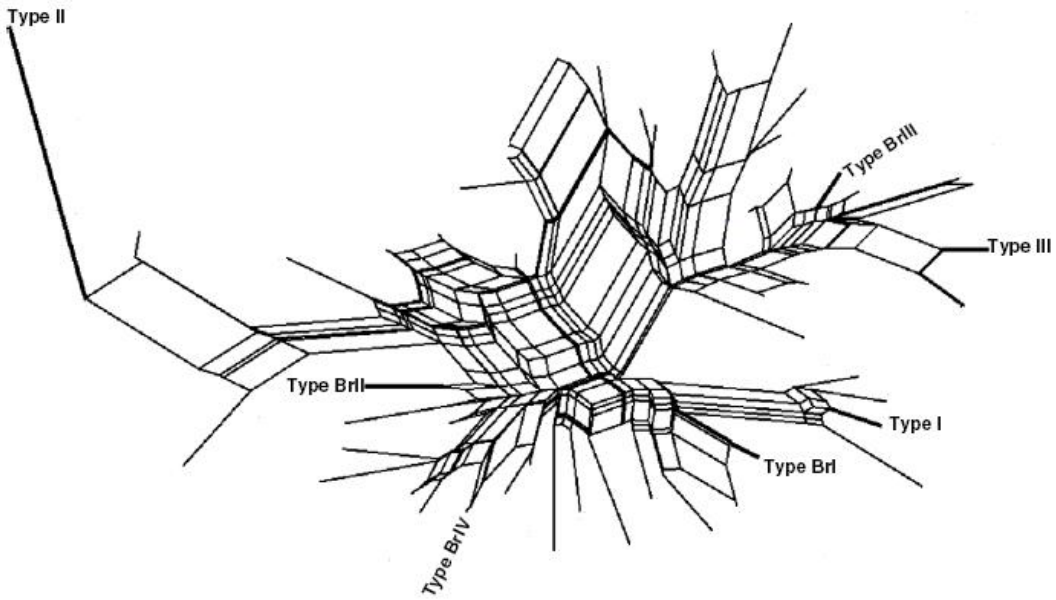


Figure 6: NeighborNet phylogenetic network of *Toxoplasma gondii* isolates from Brazil (adapted from Pena et al., 2008).

It is interesting to note that identical genotypes are found in different countries and in different hosts. This result is epidemiologically relevant, since such unchanged genotypes are obviously the product of a common clonal descent.

The greater genetic diversity of *T. gondii* in a wild and tropical environment in comparison to the domestic one is likely to be due not only to more frequent genetic exchanges, but also to the presence of SNPs and rare alleles. This greater *Toxoplasma* diversity is probably the result of the host diversity which is considerably higher in the wild host populations (Ajzenberg *et al*., 2004).

1.6.2 Molecular epidemiology: geographical distribution of genotypes

To understand molecular epidemiology of *Toxoplasma*, strains from different geographic origins should be studied. First studies on genotyping of *T. gondii* strains were limited to isolates from human and domestic animals, mainly from Europe and USA and were based on the study of a single locus. Studies based on a single marker, like *SAG2* may lead to a misidentification of atypical or recombinant strains. Study of several markers gives a more real picture of circulating genotypes, since a multilocus study identifies the strains belonging to the archetypal clonal lineages and the non archetypal strains with atypical alleles.

1.6.2.1 Domestic animals

1.6.2.1.1 Europe

Eight isolates, obtained from ewes from France were all type II by microsatellite analysis (*TUB2, TgM-A, W35, B17* and *B18*) (Dumètre *et al*., 2006). A PCR-RFLP analysis for the loci *SAG2, SAG3, GRA6* and *BTUB* revealed genotype II for 22 isolates obtained from cat feaces from Germany, Austria, France and Switzerland (Schares *et al*., 2008). Fifteen isolates were obtained from pigs from Portugal. Type II and type III were the genotypes found by *SAG2* locus and microsatelites analysis. One isolate had an atypical allele for *W35* marker (de Sousa *et al*., 2006). One isolate was obtained from a bovine aborted foetus from Azores was genotype I by *SAG2* and MS analysis (Canada *et al*., 2002; Mercier *et al*., 2006).

1.6.2.1.2 USA

In the USA an endemic avian toxoplasmosis in a farm from Illinois was also associated with type II strains. All isolates had type II alleles for the 11 studied markers (*SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, apico* and new *SAG2*) (Dubey *et al*., 2007f). Fifteen different genotypes were found in lambs for human consumption in the USA. Type II and type III clonal lineages were the most prevalent. A mixed infection and 11 atypical genotypes were also defined. Five genotypes were closely related to the clonal Type II lineage. Two different unique alleles were found for *SAG1* marker in two genotypes and a unique allele for *c22-8* and *PK1* markers in two different genotypes each (Dubey *et al*., 2008a).

1.6.2.1.3 South America

Multilocus genotyping of South American strains revealed a higher genetic diversity for *Toxoplasma* isolates. Five genotypes were found in chickens from Costa Rica: type I, type III, and three genotypes with combination of alleles I and II or alleles I and III (Dubey *et al*., 2006c). Genotypes I, II, III and four genotypes with different combination of alleles I and III were found in chickens from Nicaragua. One genotype had a unique allele for *SAG1* marker (u-1) (Dubey *et al*., 2006d). Chickens from Chile had a lower genetic diversity. Only one isolate had a combination of alleles I and III. All the other isolates were type II or type III (Dubey *et al*., 2006a). Five genotypes were defined for cats from Colombia: type I, type II and three genotypes with different associations of alleles I and III (Dubey *et al*., 2006b). Ten genotypes were revealed in dogs from Colombia that were different from Type I, II and III. A new allele (u-3) at *PK1* locus was identified in three isolates (Dubey *et al*., 2007b). One genotype found in cats from Colombia was also found in chickens from Nicaragua. Another genotype was commonly found in these four countries.

In Brazil, eighteen genotypes were found in free-ranging chickens: eleven in Para state and seven in Rio Grande do Sul. Genotypes from Para state contained different combinations of alleles I, II and III. Some genotypes had unique alleles (u-1) at loci *SAG1* and *c22-8*. Clonal types I, II and III were not found. For Rio Grande do Sul, type I and type III were found as well as combinations of allele I, II and III (Dubey *et al*., 2007e). No genotype was shared by these two geographic locations. However, one genotype found in Para state was also found in lambs from USA. Similarly, in different domestic animals from São Paulo state, Brazil, the genotypes had different combinations of allele I, II and III (Ferreira *et al*., 2006). Twenty genotypes were defined for 46 *Toxoplasma* isolates from cats from São Paulo state, Brazil (Pena *et al*., 2008). No type I, II or III lineage was found. Unique atypical alleles were found for markers *SAG1, SAG2, c22-8* and *PK1*.

1.6.2.1.4 Asia

In Asia, *SAG2* and *GRA6* type I, II and III were found in pigs from Japan. Type I and II were equally predominant, while type III was found in few samples (Zakimi *et al*., 2006a; Zakimi *et al*., 2006b). The same genotypes were found in cats from China and dogs from Vietnam (Dubey *et al*., 2007c; Dubey *et al*., 2007g). These genotypes were previously identified from dog isolates in Colombia (Dubey *et al*., 2007b), which suggests their South

America origin. Both genotypes were distinct from type I, II and III. Four genotypes were found for dogs from Sri Lanka: one type III and three unique genotypes (Dubey *et al*., 2007d). One of these unique genotypes was found also in dogs from Colombia (Dubey *et al*., 2007b) and Vietnam (Dubey *et al*., 2007c), cats from China (Dubey *et al*., 2007g) and chickens from Para, Brazil (Dubey *et al*., 2007e), indicating the widespread of this genotype. In Iran, only two genotypes were found by microsatelites analysis and *GRA6* sequencing. Type II and type III were found for isolates obtained from sheep, chickens, cats and ducks (Zia-Ali *et al*., 2007).

1.6.2.1.5 Africa

Genotype I, II and III were found for chickens from Uganda. Multiple infections were found for five chickens: three with type I and type II and two with type I, II and III (Lindström *et al*., 2007).

1.6.2.2 Wild animals

1.6.2.2.1 Europe

In Europe a type II *Toxoplasma gondii* was isolated from the brain of an adult Tawny Owl from France (Aubert *et al*., 2008b). Thirty-two isolates obtained from different wild species from France were all type II by *SAG2* locus and microsatelites analysis (Aubert *et al*., 2008a).

1.6.2.2.2 USA

Five genotypes were revealed in isolates obtained from foetal deer in USA, including the clonal type II and III lineages. Three non-clonal genotypes closely related to the clonal Type I, II and III lineages were also found (Dubey *et al*., 2008b). In sea otters two different genotypes were found, called type X (Miller *et al*., 2004) and type A (Sundar *et al*., 2008). Type X was reported for the first time in sea otters with *T. gondii*-associated meningoencephalitis (Miller *et al*., 2004). Recently, a case of disseminated toxoplasmosis resulting from transplacental infection in a neonatal sea otter from coastal California was associated with an infection by a type X strain of *T. gondii* (Miller *et al*., 2008a). The same type X was found in marine bivalves and coastal-dwelling felids from California coast (Miller

et al., 2008b). Type X is characterized by an insertion of three nucleotides (GAT) and several unique polymorphic nucleotide sites at *GRA6*, and by a unique PCR-RFLP pattern for *B1* and *SAG1* (Miller *et al*., 2004). Type A is characterized by a 15bp deletion at *GRA6* locus (Sundar *et al*., 2008).

1.6.2.2.3 South America

A *T. gondii* strain was isolated from a free-living jaguar from French Guiana. Analysis of five MS markers and *GRA6* locus revealed and atypical genotype. At *TgM-A* marker it was found an allele exclusively found in South American strains. The allele X for *GRA6* previously described in isolates from Californian sea otters was also found (Demar *et al*., 2008).

1.6.3 Genotype of *T. gondii* strains and clinical presentation of toxoplasmosis

The disease severity is related with several factors such as host, parasite and environment (reviewed by Holland, 2004). Data collected from different parts of the world suggest a different geographical distribution of the genotypes. The correlation between the pathogenesis of toxoplasmosis and the genetic characteristics of the disease-associated strains was postulated.

1.6.3.1 Congenital toxoplasmosis

According to the majority of the studies with patients from Europe and North America, type II is the genotype more often associated with congenital toxoplasmosis (Ajzenberg *et al*., 2002b; Howe and Sibley, 1995; Howe *et al*., 1997; Nowakowska *et al*., 2006; Peyron *et al*., 2006). However, Fuentes and colleagues (2001) in a study were the genetic characterization was made directly from clinical samples reported that 75% of eight congenital cases in Spain were associated with *SAG2* type I. Similar results were obtained in Colombia also directly from clinical samples, where all isolates were *SAG2* type I (Gallego *et al*., 2006). Serotyping assays in pregnant women from Colombia revealed a predominance of *GRA6* types I and III (Peyron *et al*., 2006). In Brazil, recombinant genotypes (with different combinations of alleles I, II and III) were found in congenital *Toxoplasma* infections (Ferreira

et al., 2006). Two cases of fatal congenital infections in Suriname were associated with an atypical genotype (Demar *et al*., 2007).

1.6.3.2 Immunocompromised patients

Isolates originating from immunocompromised patients are mainly type II in Europe and North America (Fuentes *et al*., 2001; Honoré *et al*., 2000; Howe and Sibley, 1995; Howe *et al*., 1997). In these regions type I and III strains seems to be less prevalent in immunocompromised patients. Howe and colleagues (1997) found *SAG2* type I in 13% of cases, and *SAG2* type III in 11%. Fuentes and colleagues (2001) found *SAG2* type I and III in 24% of cases. Honoré and colleagues (2000) found *SAG2* type I in 14.1% of cases and *SAG2* type III in 9.8%. More recently, Khan and colleagues (2005a) found a high prevalence of type I strains or strains containing type I allele in eight immunocompromised patients from North America. One isolate obtained from blood of an AIDS patient with toxoplasmosis from Iran was type III (Zia-Ali *et al*., 2007). In Colombia, *SAG2* type I strains were associated with 93.9% of 33 cases of cerebral toxoplasmosis (Gallego *et al*., 2006). Infections due to type II (11%), type III (4%) or recombinant genotypes (with type I, II and III alleles) (15%) were less prevalent than due to type I (46%) among 87 Brazilian immunocompromised patients (Ferreira *et al*., 2008). Recently, a case of severe encephalitis and chorioretinitis in an HIV African patient was associated with a I/III recombinant genotype (Genot *et al*., 2007). A study with Ugandan HIV-patients with *Toxoplasma* infection demonstrate that *SAG2* type II was the genotype more prevalent (Lindström *et al*., 2006). Fourteen genotypes were found among 88 immunocompromised patients from different geographic regions (Europe, Africa and Latin America) by the study of six microsatelites markers. Type II was the most frequent, being found in 61% of 88 patients, while type I and III were found in 2% and 11% respectively. Eight unique genotypes were found in eight patients, two genotypes were exclusively found in African patients and one genotype was characteristic of Caribbean (Ajzenberg *et al*., 2009). Type II was associated with 91.7% of 52 European patients, while non-type II genotypes were associated with 74.2% of 27 non-European patients.

1.6.3.3 Immunocompetent patients

The study performed by Grigg and colleagues (2001) in vitreous fluid of patients with severe or atypical ocular toxoplasmosis found that 41.7% of the isolates had new recombinant genotypes with alleles typical of type I or III strains at all loci examined.

Type I isolate was obtained from an ocular patient in the Republic of Korea (Chai *et al*., 2003; Lin *et al*., 2005), from patients with ocular infection in Poland (Switaj *et al*., 2006) and in ocular toxoplasmosis in humans in Brazil (Vallochi *et al*., 2005). SAG2 atypical genotype was associated with an ocular toxoplasmosis in Colombia (Gallego *et al*., 2006). Type II was found associated with two cases of acute toxoplasmosis in Iran (Zia-Ali *et al*., 2007). Atypical multilocus genotypes were found in the rare cases of severe toxoplasmosis with multivisceral involvement in immunocompetent adult patients in France, French Guiana and Suriname (Carme *et al*., 2002; Demar *et al*., 2007; De Salvador- Guillouët *et al*., 2006).

1.6.4 Limits of genotyping

Genetic studies of *T. gondii* are based on genotyping methods that require previous isolation of the parasite or of its DNA. Consequently biological samples are required. In patients with clinical signs of the disease, infected samples are relatively easy to obtain. According to the disease, parasite or its DNA can be isolated from tissue samples such as placenta, amniotic liquid, ocular and cerebrospinal fluid. However, biological samples for bioassay are not always available, especially from asymptomatic patients and isolates are not obtained from all biological samples.

In summary, the genetic population structure of *T. gondii* is more complex and genetic diversity higher than initially described. Study of *Toxoplasma* strains from different geographic regions is needed to understand the *Toxoplasma* geographic distribution. Actually, available data point to a prevalence of the archetypal clonal lineages (I, II, III) in Europe and North America. In South America, *Toxoplasma* population has a high genetic diversity, with unique atypical alleles being found. The genotype responsible for *Toxoplasma* infections (both symptomatic and asymptomatic) seems to be determined by the geographical origin of the infection. Technical development and new genetic markers are essential for the knowledge of *Toxoplasma* genetic diversity.

1.7 Serotyping

A new genotyping approach was developed based on the antibody-antigen specific recognition. This principle was already applied for the genetic characterization of other microbiologic agents (Dou *et al.*, 2007; Simon *et al.*, 2001; Walker *et al.*, 2006; Wu *et al.*, 2003). It was developed by Kong *et al* (2003), and basically consists in an enzyme-linked immunosorbent assay using synthetic polymorphic peptides from *Toxoplasma* immunogens to detect strain-specific antibodies. Synthetic peptides were coupled at the N or the C-terminal to the carrier protein keyhole limpet hemocyanin. Several peptides selected from a variety of antigens were tested (GRA, SAG, ROP, NTP, BSR and SRS proteins). Only peptides derived from SAG2, GRA3, GRA6 and GRA7 could differentiate *Toxoplasma* strains in infected mice. Results obtained in mice were not totally reproduced in humans. From these four antigens, only the peptides derived from GRA6 and GRA7 were able to differentiate *Toxoplasma* strains in human sera (Table 5). However, the peptides could only differentiate type II from non-type II infections.

Table 5: Peptides tested by Kong and colleagues (2003). Polymorphic aa in red.

Peptide	Sequence	Reactivity
GRA6-I/III-220	CLHPERVNVFDY	Strongly recognized
dGRAS6-I/111-220	CLHPERVNVFD	Strongly recognized
dGRAS6-III-220(9)	CLHPERVNV	Strongly recognized
GRA6-II-214	CLHPGSVNEFDF	Strongly recognized
dGRA6-II-214	CLHPGSVNEFD	Strongly recognized
dGRA6-II-216(9)	C——PGSVNEFDF	Strongly recognized
dGRA6-II-214(9)	CLHPGSVNE	Strongly recognized
GRA3-II-28	ADQPGNHQALAEPVC	Strongly recognized

Peptide	Sequence	Reactivity
GRA7-II-225	CVPESGKDGEDARQ	Strongly recognized
dGRA7-II-225	CVPESGKDGEDA	Strongly recognized
GRA7-III-225	CVPESGEDREDARQ	Strongly recognized
dGRA7-III-225	CVPESGEDREDA	Strongly recognized
SAG2A-I/III-131	PAGRNND—GSSAPTPKC	Strongly recognized
dSAG2A-I/III-131(13)	PAGRNND—GSSAPC	Strongly recognized
dSAG2A-I/III-134(10)	RNND—GSSAPC	Strongly recognized
SAG2A-II-131	PAGRNNDGGSSAPTPKC	Strongly recognized
dSAG2A-II-134(11)	RNNDGGSSAPC	Strongly recognized
GRA6-I/III-175	CGRRSPPERSGDGG	Recognized
GRA6-II-175	CGRRSPQERSGGGG	Recognized
GRA6-I/III-199	CGNEGRGYGGRGEG	Recognized
GRA6-I-207	CGRGEGGAEDDRRP	Recognized
GRA6-II-202	CGRGEGG—EDDRRPL	Recognized
ROP1-I-85	PVRGPDQVPAC	Recognized
ROP1-II/III-85	PVRDPRQVPGRGEC	Recognized
ROP1-II/111-359	CTRVRGALR—GRGR	Recognized
GRA7-I-163	ELTEEQQRGDEPLC	Recognized
dGRA7-I-162	CPELTEEQQRG	Recognized
GRA7-I/II-215	CSRQPALEQEVPES	Recognized
GRA7-III-215	CSRQPAPEHEVPES	Recognized

Peptide	Sequence	Reactivity
GRA7II-225	CVPESGEDGEDARQ	Recognized
SAG3-II-49	GNSRRKITYC	Recognized
NTP3-I-99	SIQLIGAGKRFAGLRC	Not recognized
NTP1-II/III-99	SIRLIREGKRFTGLRC	Not recognized
NTP1-I/II/III-485	CAPMFITGREMLASIDT	Not recognized
NTP3-I-485	CAPMIVTGGGMLAAINT	Not recognized
BSR4-I/II-155	KVNEQREESNKSQKC	Not recognized
BSR4-I/II-336	PKKDKESGTETGAPC	Not recognized
SAG1-174	SYGADSTLGPVKLC	Not recognized
SAG1-I-244	SDKGATLTIKKEAFPC	Not recognized
SAG2A-I/III-88	PGAVLTAKVQQPAKGPC	Not recognized
SAG3-II-120	CHIDAKDQDD	Not recognized
SAG4A-I/II-84	CKDEPVELAAL	Not recognized
SRS1-I-50	SMTSPLLTWDGNKVTC	Not recognized
SRS2-I-53	GPPYRYEPEKFTC	Not recognized
GRA1-I-92	CSYSEVGNVNVEE	Not recognized
GRA1-III-92	CSYSEVGDVNVEE	Not recognized
GRA1-I/III-159	CQDEMKVIDDVQQ	Not recognized
GRA1-II-159	CQDEMNVIDDVQQ	Not recognized
GRA1-II-159	SAAIGGRMVSRTLRDNIPGC	Not recognized
GRA3-I/III-189	RRKPKDEGAGVDKAC	Not recognized

Peptide	Sequence	Reactivity
GRA4-I-232	CTEDSGLTGVKDSSS	Not recognized
ROP1-I-131	NSEDDDTFHDAC	Not recognized
ROP1-II/III-131	NSEDD—TFHDAC	Not recognized
ROP1-II/III-181	QELPPPNAQELC	Not recognized

A similar approach was proposed by Peyron and colleagues (2006), but with recombinant polymorphic peptides of GRA5 and GRA6 antigens, specific for type I, II and III strains. Peptides were derived from the hydrophilic N-terminal region of GRA5 (the first 75 aa). In that region are found all but one polymorphic residues that distinguishes the types I, II and III. GRA6 C-terminal region selected by Peyron and colleagues (2006) comprised position 172 to 230. The N-terminal portion of GRA5 and the C-terminal portion of GRA6 were expressed as polypeptides fused to GST using the pGEX-3X vector l. Peptides specific for GRA5 and GRA6 type II strains were able to differentiate type II strains from non-type II. Peptides specific for types I and III strains cross-reacted and could not differentiate these two strain types.

The aim of the present study is to develop serotyping by selecting peptides that could differentiate the three clonal lineages (I, II and III) as well as the non-archetypal strains. Both previous studies selected GRA proteins for serotyping of human serum samples. Moreover, the study performed by Kong and colleagues (2003) showed that polymorphic peptides derived from predicted immunogenic regions from other *T. gondii* antigens were not reactive or poorly recognized by human sera. Based on these previous results, three GRA antigens (GRA6, GRA7 and GRA8) were selected for peptide design in the present study. Peptides derived from GRA6 antigen were described by both authors as able to discriminate strains and studies had demonstrated that GRA6 is a very polymorphic locus (Fazaeli *et al*., 2000). This characteristic can be useful to define peptides specific for the atypical strains. GRA7 peptides were also able to differentiate strains, especially type II and III. GRA8 was chosen, because this antigen is immunogenic (Beghetto *et al*., 2003) and possible polymorphic. Sequencing of

a *T. gondii* strain from Korea revealed that GRA8 possess two nucleotide and two amino acids substitutions when compared with RH strain (Lin *et al*., 2005). Peptides described for GRA5 have not a high discriminative power and based on that this antigen was not included in this study.

Toxoplasma gondii is a parasitic zoonosis with a worldwide distribution. This successful parasite is capable of infecting all warm-blooded animals. Its complex life cycle is responsible for the success of this parasite in different hosts around the world.

The majority of human infections are asymptomatic. However, severe clinical complications may occur as a consequence of a congenital infection or as opportunistic infections in immunocompromised patients. Immunocompetent patients, which are usually asymptomatic for toxoplasmosis, may also develop severe *Toxoplasma*-related disease. Recently, in remote tropical areas, new cases of severe toxoplasmosis have occurred in immunocompetent patients presenting unsuspected clinical features, namely with the involvement of pulmonary complications and even death. The genetic characterization of *T. gondii* associated with these cases revealed the emergence of highly virulent non archetypal genotypes strains different from the archetypal lineages. In order to understand the pathogenesis of toxoplasmosis it is necessary to proceed to the genetic characterization of the *T. gondii* associated with chronic and acute infections.

Development of genetic markers was essential for the advances in the knowledge of *Toxoplasma* genetic diversity. However, genotyping methods have some limitations and may introduce bias on the genetic studies. Limits are related with parasite isolation, since genotyping methods are based on DNA analysis. Biological samples needed for parasite isolation are difficult to obtain, especially from asymptomatic patients. Development of a typing method not based on DNA studies would eliminate the limits inherited to genotyping methods. Serotyping, being a method based on strain specific antigen-antibody recognition does not require isolation of the parasite. This method seems to be a promising tool for typing *Toxoplasma* strains.

Two main objectives were defined to this work. First, explore serotyping as a typing method by defining polymorphic peptides specific for the different types of strains derived from GRA proteins. These proteins are immunogenic, polymorphic, and previous studies showed that peptides derived from these antigens have a higher sensitivity and specificity than peptides derived from other antigenic proteins. Second, use the established serotyping protocol to study the genetic diversity of *T. gondii* according to geographic origin and human disease.

Part II

Material and Methods

2.1 Selection of peptides

The first objective of this work was to define polymorphic peptides specific for the three clonal lineages (I, II, III) and for the non-archetypal strains. Two *Toxoplasma* antigens (GRA6 and GRA7) were selected based on previous serotyping studies (Kong *et al*., 2003; Peyron *et al*., 2006). GRA8 was selected based on its immunogenic properties. These three antigens from archetypal and non-archetypal *Toxoplasma* strains were sequenced to define polymorphic regions.

2.1.1 *Toxoplasma gondii* strains

Fifty two strains of *T. gondii* were selected. RH, BEVERLEY and NED were used as type I, II and III reference strains respectively. The remaining 49 strains were selected based on a previous genotyping study using five MS markers (*TUB2, W35, TgM-A, B18, B17*) showing that they differed from the archetypal type I, II or III. The isolates TgCkCo24, GANGI and ENVL-2002-MAC were considered as variant of type I, II and III respectively, because of minor variations in their genotypes in comparison with the three archetypal clonal lineages (Appendix 2). The isolates CRL-2004-MOT and IPB-2003-COG with different combinations of type I, II or III alleles were considered as mixed III/I and II/III genotypes respectively (Appendix 2). The other isolates were atypical because of the presence of atypical alleles in their genotypes. The MS genotype of 15 strains has already been published (Ajzenberg *et al*., 2004). Thirty seven strains originated from South America or the Caribbean Islands, 6 from Africa, 1 from the Indian Ocean, 5 from Europe, and 3 from USA (Appendix 2).

2.1.2 DNA extraction

The DNA was extracted from infected mouse brain using the QIAmp DNA Mini Kit (Quiagen, Courtaboeuf, France), according to the manufacturer's protocol.

Infected mouse brain tissue was previously homogenized in PBS. To 100 µl of the brain homogenate, 100 µl of Buffer ATL, and 20 µl of Proteinase K were added. The mixture was incubated at 56ºC for 1 h until the tissue was completely lysed. Buffer AL (200 µl) was added and a second incubation step at 70ºC for 10 min was performed. Absolute ethanol (200 µl) was added and the mixture was transferred to the QIAmp Spin Column (in a 2 ml collection tube), and centrifuged at 8000 rpm for 1 min. The mixture was washed with 500 µl washing buffer AW1 (centrifugation at 8000 rpm for 1 min) and with 500 µl washing buffer AW2 (centrifugation at 14000 rpm for 3 min). Another centrifugation step at 14000rpm was performed for 1 min to remove residual washing buffer AW2. Finally an elution step was made by adding 200 µl of elution buffer, followed by incubation at room temperature for 5 min and centrifugation at 8000 rpm for 1 min.

2.1.3 Sequencing

The coding regions of *GRA6, GRA7* and *GRA8* genes were amplified by PCR reactions using the following primers (Table 6).

Table 6: Primer sequences for *GRA6, GRA7* and *GRA8* sequencing

Locus		Primer sequence	Reference
GRA6	forward	5'-GTAGCGTGCTTGTTGGCGAC-3'	Fazaeli *et al*., 2000
	reverse	5'-TACAAGACATAGAGTGCCCC-3'	Fazaeli *et al*., 2000
GRA7	forward	5'- ACCCTATATTGGGGCTTGCT-3'	Marle-Plistat, 2005
	reverse	5'-ACACTGTCCTCGAGCTCCTA-3'	Marle-Plistat, 2005
GRA8	forward	5'-ATGGCTTTACCATTGCGTG-3'	Lin *et al*., 2005
	reverse	5'-TTAATTCTGCGTCGTTACGG-3'	Lin *et al*., 2005

The PCR mix was composed by the Qiagen PCR Multiplex Kit (Qiagen, Courtaboeuf, France) with 2X Qiagen Multiplex PCR master mix at a final concentration of 1X, 1µM of each primer and 4 µl of DNA, in a total volume of 25 µl.

The DNA was amplified using the following conditions: one cycle for initial denaturation of 15 min at 95°C, 35 cycles of 94°C for 30 s, 65°C (GRA6)/63°C (GRA7)/ 60ºC (GRA8) for 3 min, 72°C for 1 min, and a final extension step at 60°C for 30 min.

Amplifications were carried out in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems). The PCR products were visualized on a 2% agarose gel under UV light.

PCR products were purified using the CONCERT Rapid PCR Purification System kit (Marligen Biosciences, Montrouge, France), according to manufacturer's protocol. PCR products were mixed with 400 µl of binding solution and vortexed. The sample was loaded into the cartridge (supplied by the kit) and centrifuged at 12000 x g for 1 min. The flow-through was discarded. The filter was washed with 700 µl of washing buffer at 12000 x g for 1 min. A second centrifugation was done at 12000x g for 1 min to remove all residual washing buffer. The purified DNA was recovered by adding 50 µl of elution buffer warm to 65°C to the filter, incubated at room temperature and then centrifuged at 12000 x g for 2 min.

The purified PCR product was directly sequenced in both directions. The mix for sequencing was composed by 2.7 µl ABI PRISM BigDye terminator cycle sequencing kit (Applied Biosystems), 0.4 µM of primer, and 4 µl of purified DNA in a total volume of 10 µl. The purified DNA was amplified using the following conditions: one cycle for initial denaturation of 1 min at 96°C, 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Amplification products were left in 2-propanol (60 µl of 2-propanol and 30 µl of H_2Od) at dark overnight for DNA precipitation. Precipitated DNA was centrifuged at 14500rpm for 20 min to remove 2-propanol. The DNA was washed with 250 µl of ethanol 70% at 14500rpm for 5 min. After removal of ethanol, 15 µl of formamide on ice was added and the DNA was denaturated at 95°C for 3min and run in a ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.1.4 Sequence analysis

The sequences were aligned with type I (RH), type II (BEVERLEY) and type III (NED) reference strains using the CLUSTALW software (http://www.ebi.ac.uk/Tools/clustalw/). The nucleotide translation was made with the ExPASy - Translate tool software (http://www.expasy.ch/tools/dna.html). Haplotypes were defined with DnaSP 4.20.2 for Windows.

2.1.5 Antigenic epitopes prediction

The prediction of antigenic epitopes was made with the ExPASy – ProtScale software (www.expasy.org/tools/protscale.html) based on three parameters; (i) hydrophobicity (Eisenberg *et al*., 1984), (ii) hydrophilicity (Hopp and Woods, 1981) and (iii) antigenicity (Welling *et al*., 1985). MIF Bioinformatic: predicted antigenic peptides software (http://immunax.dfci.havard.edu/tools/antigenic.html) was also used to predict potential antigenic sites.

2.2 Phylogenetic study

A phylogenetic study based on GRA6 and GRA7 nucleotide sequences was performed using MEGA 4.0.2 software. The Molecular Evolutionary Genetics Analysis (MEGA) software was designed for comparative analysis of homologous gene sequences with a special emphasis on inferring evolutionary relationships and patterns of DNA and protein evolution (Kumar *et al*., 2008). Phylogenetic tree was inferred using the Neighbor-Joining method. The Neighbor-Joining method produces a tree under the principle of minimum evolution. The principle of this method is to find pairs of "neighbors" that minimize the total branch length at each stage of clustering of operational taxonomic units. A pair of neighbors is a pair of operational taxonomic units connected through a single interior node in an unrooted bifurcating tree (Saitou and Nei, 1987).

2.3 Experimental infection with known genotypes

Experimental infections in mice were performed in order to obtain serum samples from controlled known infections. These sera will be used to evaluate the usefulness of the selected peptides to distinguish the different types of *T. gondii* strains.

2.3.1 Laboratory animals

For experimental infections with *T. gondii* strains, CD1 mice were used. Mice of 2 months old were maintained with periods of 12h light and 12h dark, at 22°C, with food and water *ad libitum.*

2.3.2 Experimental infections

Eighteen CD1 mice were divided in six groups of 3 mice each (group A to F).

Mice from groups A to D were inoculated with cysts of the following strains:

- Group A - PT-2005-SUS SCR 05 (type III strain)
- Group B - PT-2005-SUS SCR 16 (type III strain)
- Group C - MAR-2000-HOU (recombinant III/II/I strain)
- Group D - GUY-2004-AKO (atypical strain)

Mice from group E were inoculated with oocysts of the atypical strain TgCkAr23.

Mice from group F were immunized with type I RH strain (tachyzoites from RH strain were frozen at -80°C for 1h30min, boiled at 100°C for 5 min and sonicated 3 times (2 min/cycle) to induce tachyzoite lyse). A second immunization with the same RH strain lysate was performed one week later using incomplete Freund adjuvant (1:1).

Mice from groups A to E were treated with sulphadiazine during 10 days post-inoculation to avoid animal death during acute phase of infection.

Four weeks later, mice were bled and *Toxoplasma* antibodies were detected by MAT. Mice were considered seropositive at a dilution equal or higher than 1/40.

2.3.3 Modifief Agglutination Test protocol

Antibodies against *T. gondii* in experimentally infected mice were detected by MAT according the protocol established by Desmonts and Remington (1980). *Toxoplasma* antigen was obtained from peritoneal wash of mice infected with RH strain of *T. gondii* together with TG 180 sarcoma cells. The tachyzoites were treated with trypsin, and then fixed with formalin. The modified agglutination test measures only IgG antibodies, because the 2-mercaptoethanol destroys specific and non-specific IgM antibodies.

A sequential dilution of serum in 100 µl of PBS/2-mercaptoethanol (1.4%) is made on a microplate of 96 wells, followed by addition of 50 µl of formalized antigen diluted in BABS (1/20). The mixture is incubated overnight at 37ºC in a moist atmosphere. Results are obtained by microscopic observation of agglutination.

2.4 Serum samples

Serum samples from experimentally infected mice and from domestic animals and humans naturally infected with *T. gondii* with known genotype were selected in order to evaluate the usefulness of the defined peptides for serotyping and validate the serotyping protocol. Non-genotyped infections from naturally infected domestic animals and humans were used to predict geographic serotype distribution and possible association with disease manifestation.

2.4.1 Mice

Fifty-four serum samples from forty-five animals were tested. Serum samples from type I, III and atypical infections were obtained from experimental infected mice (group A to F) that seroconvert. Mice experimentally infected were bled four and eight weeks post-infection. One serum sample from a type III infection was collected from one mouse chronically infected with the strain PT-2005-SUS SCR 02. Type II serum samples were obtained from 27 mice chronically infected with type II strains isolated from chickens and humans. Serum samples from thirty negative control mice were use to establish cut-off values (Table 7).

2.4.2 Domestic animals

Naturally infected domestic animals (chickens, pigs and sheep) from Portugal were selected.

Chickens and pigs were selected from a study of genetic characterization of the *Toxoplasma* Portuguese population (de Sousa *et al.*, 2006; Dubey *et al.*, 2006e). Eleven isolates were obtained from chickens and fifteen were obtained from pigs. Isolates from chickens were genotyped by the study of *SAG2* locus. Four out of eleven isolates obtained from chickens were also genotyped by five MS markers (*BTUB, TgM A, W35, B17, B18*). Isolates from pigs were genotyped by the same five MS markers. Eight isolates from chickens

were type II and three were type III. Ten isolates from pigs were type II, one was type II* (* indicate the existence of an atypical allele for the MS marker, *W35*) and the remaining four were type III. Serum samples from these animals were selected.

Thirty-five serum samples from chickens, twenty-nine from pigs and fifty serum samples from sheep positive for *T. gondii* but from which no isolate was obtained were also studied.

Serum samples from 13 *Toxoplasma* negative chickens, 10 *Toxoplasma* negative pigs and 16 *Toxoplasma* negative sheep were used to establish cut-off values (Table 7).

Table 7: Summary of serum samples from mice and domestic animals

	Genotype				**Unknown genotype**	**Negative**
	I	**II**	**III**	**Atypical**		
Mice	5	27	9	13	0	30
Chickens	0	8	3	0	35	13
Pigs	0	11	4	0	29	10
Sheep	0	0	0	0	50	16

2.4.3 Humans

Three distinct geographical areas were selected for this study: Europe, Africa and Latin America. Human serum samples from Europe were collected in France and Portugal, those from Africa were collected in patients originating from Ivory Cost, Congo, Angola, Cameroon and Gabon, and those from Latin America were collected in French Guiana, Suriname, Colombia, Mexico and Uruguay.

Human serum samples related to 28 strains from the *Toxoplasma* bank of the Biological Resource Center for Toxoplasma group were selected. The strains, previously typed by the analysis of 5 MS markers (*TUB2, TgM-A, W35, B17, B18*) as described by

Ajzenberg and colleagues (2005), had the following genotypes: (i) 2 cases associated with type I (lymphadenopathy following a laboratory accidental infection with RH strain); (ii) 19 type II strains (18 from congenital infections and one isolated from the tonsils of a patient); (iii) 3 type III from congenital infections; (iv) 16 cases associated with 5 atypical strains (12 of them caused by a single strain isolated during a *Toxoplasma* outbreak in Suriname) (Demar *et al*., 2007).

Human serum samples from 672 *Toxoplasma* symptomatic and asymptomatic infections with unknown genotype were grouped according to geographical origin (Table 8).

A) Serum samples from 221 patients from Europe: (i) 94 from France; (ii) 126 from Portugal and (iii) 1 from Denmark.

B) Serum samples from 86 patients from Africa: (i) 17 from Congo; (ii) 6 from Angola (iii) 5 from Gabon; (iv) 38 from Ivory Coast and (v) 20 from patients with undetermined African origin.

C) Serum samples from 365 patients from Latin America: (i) 31 from French Guiana (Pr Bernard Carme, Cayenne); (ii) 124 from Mexico; (iii) 2 from Colombia (Dr Jorge Gomez, Armenia) and (iv) 208 from Uruguay (Dr Andres Puime, Montevideo).

To establish cut-off values, 128 *T. gondii* negative human sera were selected from the following countries: France (29 sera), Portugal (17 sera), Mexico (22 sera), Suriname (21 sera), French Guiana (5 sera), Uruguay (12 sera) and Ivory Cost (22 sera).

Table 8: Summary of serum samples from humans

	Genotype				**Unknown genotype**	**Negative**
	I	**II**	**III**	**Atypical**		
Europe	6	35	6	0	221	46
Africa	0	0	0	0	86	22
Latin America	0	0	0	16	365	60
Total	6	35	6	16	672	128

2.5 Serotyping protocol

A typing method based on an immunoenzymatic assay was developed using polymorphic synthetic peptides as antigen.

2.5.1 Synthetic peptides

Strain-specific peptides derived from GRA6, GRA7 and a peptide control, were used. Peptides were selected based on strain-specific polymorphisms and antigenic epitopes prediction results. The single aa sequence of the selected peptides varied from 11 to 33 residues. For each sequence, two peptides were synthesized: the single sequence of aa and repeats of the single sequence in the same peptide. For sequences with less than 20 amino acids, the selected region was repeated three times in one single peptide. For larger sequences, the selected region was repeated twice in one single peptide. Two peptides derived from GRA6 and two derived from GRA7 were defined for archetypal strains. Two peptides derived from GRA6 and one derived from GRA7 were defined for non-archetypal strains. Control peptide and the peptides specific for the archetypal strains derived from GRA6 antigen were based on the peptides described by Kong and colleagues (2003). The control peptide (EVVHDYRLFNPEVVHDYRLFNPEVVHDYRLFNP) derives from the C-terminal region of GRA6 antigen specific of type I and type III strains (between aa 220 and 230) by randomizing the sequence. Peptides were synthesized (Bachem, Suisse and Millegen, France) without carrier protein, since plates specific for peptide coupling were used.

In order to define the ELISA conditions, several assays were performed, where the different variables (serum and conjugate concentration, incubation time, washing conditions, dilution buffer) were tested.

2.5.2 ELISA protocol

2.5.2.1 Mice serum samples

Immobilizer amino plates (Nunc, Denmark) were coated with each peptide diluted to 10 μg/ml in 0.05M carbonate/bicarbonate buffer pH 9.6 by incubation overnight at 4ºC. Wells were blocked with a solution of 5% milk in PBS for 1h at 37ºC in a moisture atmosphere and then washed 3 times with 0.3% Tween 20 in PBS. Sera were diluted to 1/100 in 0.3% Tween 20 in PBS and incubated for 2h at 37ºC in a moisture atmosphere. Wells were washed 3 times with 0.3% Tween 20 in PBS. Anti-mouse IgG HRP conjugate (Dako, USA) was diluted at 1/4000 in 0.3% Tween 20 in PBS and incubated for 1h at 37ºC in a moisture atmosphere. Wells were washed 3 times with 0.3% Tween 20 in PBS and developed with o-phenylenediamine (OPD) for 15 minutes at 37ºC. Reaction was stopped with HCL 3M, and absorbance was measured at 490nm.

Specific conjugates were used for mice IgG isotypes. Anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b and anti-mouse IgG3 were diluted at 1/4000 in 0.3% Tween 20 in PBS and incubated for 1h in a moisture atmosphere. The wells were washed 3 times with 0.3% Tween 20 in PBS. Donkey anti-sheep/goat peroxidase antibody (Binding Site) was diluted to 1/1000 in 0.3% Tween 20 in PBS and incubated for 1h in a moisture atmosphere.

2.5.2.2 Chicken serum samples

Immobilizer amino plates (Nunc, Denmark) were coated with each peptide diluted to 10 μg/ml in 0.05M carbonate/bicarbonate buffer pH 9.6 by incubation overnight at 4ºC. Wells were blocked with a solution of 3% BSA in PBS for 1h at 37ºC in a moisture atmosphere and then washed 3 times with 0.3% Tween 20 in PBS. Sera were diluted to 1/800 in a solution of 3% BSA in PBS with 0.3% Tween 20 and incubated for 2h at 37ºC in a moisture atmosphere. Wells were washed 3 times with 0.3% Tween 20 in PBS. Anti-chicken IgG peroxidase labeled conjugate (KPL, USA) was diluted at 1/32000 in a solution of 3% BSA in PBS with 0.3% Tween 20 and incubated for 1h at 37ºC in a moisture atmosphere. Wells were washed 3 times with 0.3% Tween 20 in PBS and developed with OPD for 15 minutes at 37ºC. Reaction was stopped with HCL 3M, and absorbance was measured at 490nm.

2.5.2.3 Pig serum samples

ELISA protocol was the same followed for chickens with the following changes. No Tween 20 was used for sera and conjugate dilutions, according to conjugate manufacturer's recommendations. Serum samples were diluted at 1/100 in a solution of 3% BSA in PBS and the conjugate anti-swine IgG peroxidase labeled (KPL, USA) was diluted at 1/1000 in a solution of 3% BSA in PBS. Reaction was developed with OPD for 15 minutes and stopped with HCL 3M. Absorbance was measured at 490nm.

2.5.2.4 Sheep serum samples

ELISA protocol was the same followed for chickens with the following changes. Sheep serum samples were diluted to 1/100 in a solution of 3% BSA in PBS with 0.3% Tween 20 and the anti-sheep/goat IgG peroxidase labeled conjugate (The Binding Site, UK) was diluted at 1/10000 in a solution of 3% BSA in PBS with 0.3% Tween 20. Reaction was developed with OPD for 15 minutes and stopped with HCL 3M. Absorbance was measured at 490nm.

2.5.2.5 Human serum samples

ELISA protocol was the same followed for chickens with the following changes. Human serum samples were diluted to 1/50 in a solution of 3% BSA in PBS with 0.3% Tween 20 and the conjugate anti-human IgG alkaline phosphatase (Pierce, USA) was diluted at 1/5000 in a solution of 3% BSA in PBS with 0.3% Tween 20. Reaction was developed with p-nitro phenyl phosphate for 15 minutes at 37ºC. Absorbance was measured at 415nm.

Optical density (OD) values were calculated by subtracting the OD of the peptide control from the OD of each peptide. For humans, different cut-off values were calculated for the three geographical regions. Serum samples negative for *T. gondii* were used to calculate the cut-off. Cut off was set on the mean absorbance readings of negative sera plus 2SD (standard deviation).

2.6 Statistical analysis

Statistical analysis was performed using SPSS Version 12.0 for Windows. The Chi-squared test was performed to assess the statistical significance of differences in the prevalence of serotypes for different geographical regions and for different pathologies. *p* Values of less than 0.05 were considered significant.

Part III

Results

3.1 Sequence analysis of *Toxoplasma gondii* strains

3.1.1 *GRA6* sequencing

The alignment of 49 atypical strains and of the 3 reference strains showed 52 polymorphic positions at nucleotide level. Nineteen different haplotypes were identified (Appendix 3 - Tables 1 and 2). In comparison with RH strain, one deletion of 15bp (from nucleotide 635 to nucleotide 649 for haplotypes 2 and 7) and one deletion of 3bp (haplotypes 2, 4, 12 and 19 from nucleotide 658 to 660) were found. One insertion of 3bp was found for strain GUY-2004-JAG (Appendix 3 - Table 2). Guanine (G) at position 71 and adenine (A) at position 304 found in haplotype 1 was replaced by a thymine (T) in the remaining haplotypes. Haplotypes 2, 4, 15 and 19 have a T at position 41, a C at position 336 and a A at position 702. Haplotypes 5, 6, 7, 14 and 17 have a A at positions 162 and 692. Eight strains shared the sequences described in type I reference strain RH (haplotype 1), type II reference strain BEVERLEY (haplotype 2) or type III reference strain NED (haplotype 3). The variant of type I (TgCkCo24) and RH strain have the same GRA6 sequence. BEVERLEY strain sequence was found for the variant of type II (GANGI) and for three atypical strains (TgCkBr11, TgCkBr25 and TgCkBr10). NED sequence was found in the variant of type III (ENVL-2002-MAC) and for two strains with mixed III/I and II/III genotypes (CRL-2004-MOT and IPB-2003-COG respectively). The haplotypes 6, 8, 9, 13 and 15 were only found in strains from French Guiana. Most African strains share the haplotype 11. Haplotype 10 was found in eight of 23 Brazilian isolates and in one isolate from French Guiana (Appendix 3 - Table 1).

3.1.2 Selection of polymorphic peptides from GRA6 antigen

GRA6 locus codes for a protein of 230 aa. At amino acid level, one insertion was found at position 91 (D, Aspartate) for GUY-2004-JAG. Five amino acids were deleted in the haplotype 7 (aa 204 to aa 208), and one in the haplotypes 4, 12 and 19 (position 213) (Appendix 3 - Table 3). These deletions are also shared by BEVERLEY (type II). All the nucleotide sequences code for a specific sequence of amino acids, except the haplotypes 10 and 18 that share the same sequence of amino acids.

The most polymorphic region at GRA6 protein sequence is the C-terminal region. At this region (aa 198 to aa 230), 15 different peptides can be selected that allow to differentiate *Toxoplasma* strains (Table 9). Peptides corresponding to type I (haplotype 1) and type III (haplotype 3) strains differed from each other by 3 aa substitutions (positions 198, 213, 219) and are clearly different from peptide corresponding to type II (haplotype 2) strains characterized by deletions from aa 204 to 208, and at position 213, and by 4 to 5 aa substitution from aa 219 to 230. The remaining 12 peptides are found in most of the non-archetypal strains (Table 9) circulating in South America or Africa. They differed from type I or III peptides by 1 to 3 aa substitutions. Peptide corresponding to haplotypes 8 and 9 (G–GYRGR–G–DRRA–ER–V–Y) is found in four of 10 French Guiana strains. Peptide corresponding to haplotypes 10 and 18 (G–GYRGR–G–DRRA–EH–V–Y) is found in eight of 23 Brazilian strains. These two peptides differ from each other by a single aa (arginine instead of histidine at position 224) and from haplotype 1 or 3 by 2 to 4 aa substitutions. Peptide corresponding to haplotypes 11 and 13 (G–GYRGR–G–DGRA–ER–V–Y) is found in four of the six African strains. Peptides ([R–GYGGR–G–DRRA–EH–V–Y], [G–GYRGR–G–DRRA–ES–V–Y], [G–GYGGR–DRRP–ER–E–Y] and [G–GDGGR–G–DRRP–ER–E–Y]) are exclusive from South-American strains.

The regions of the GRA6 protein between position 47 and 58, between position 92 and 105 and between positions 180-190, also show polymorphisms that can differentiate some atypical strains (Table 9). However in these regions, most of the polymorphisms present in atypical strains are shared by the types I, II or III. Between position 47 and 58, five different peptides can be selected. Peptides [G-VK-], [G-VR-] and [D-VK-] correspond to haplotypes 1, 2 and 3 respectively. However, these peptides are also shared by atypical strains. Peptide [G-VK-] is found in one strain from USA, one from France, one from Colombia, six of the ten strains from French Guiana and in twelve of the 23 Brazilian strains. Peptide [D-VK-] is found in ten South American or Caribbean strains, three European strains, one from the United States, one from Africa and one from Reunion Island. Besides strains belonging to haplotype 2, peptide [G-VR-] is also found in GUY-2004-JAG (haplotype 15). Peptide [G-VQ-], characteristic of haplotypes 11 and 12 is found in four of the six African strains. Peptide [G-FR-] is exclusive of the Brazilian TgCkBr2 strain (haplotype19). Five different peptides were found between positions 92 and 105. Peptide [T-E-V] is characteristic of haplotype 1. Peptide [A-D-A] is exclusive of strain GUY-2004-JAG. Peptide [T-D-A] is

found in strains from haplotypes 2, 4 and 19. Haplotype 3 shares the peptide [T-D-V] with the haplotypes 5, 8, 9, 10, 11, 12, 13, 16, 17 and 18. Peptide [A-D-V] is found in haplotypes 6, 7 and 14. Between positions 180 and 190, peptides [Q-DG-D] and [Q-DG-G] are exclusively found for haplotype 1 strains and in the strain TgCkBr2 (haplotype 19) respectively. Peptide [Q-GG-D] is found in strains from haplotype 2, 4 and 15. Haplotype 3 shares the peptide [P-DG-D] with the strains belonging to the haplotypes 5, 11, 12, 16 and 17. Peptide [P-DD-D] is found in 21 of the 37 South American strains.

Table 9: Polymorphic peptides from *GRA6* marker. Periods (.) indicate identical amino acid and dashes (-) indicate deletion.

Position	Polymorphic peptide	Strain type
47-58	GGVKQTPSETGS	I, Atypical
	G . VR	II, Atypical
	D . VK	III, Atypical
	G . VQ	Atypical
	G . FR	Atypical
92-105	TSEAAEGDVDPFPV	I
	T . D . . . . . A	II, Atypical
	T . D V	III, Atypical
	A . D V	Atypical
	A . D . . . . . A	Atypical
180-190	QEPSGDGGGND	I
	Q . . . . GG . . . D	II, Atypical
	P . . . . DG . . . D	III, Atypical

Position	Polymorphic peptide	Strain type
180-190	P . . . . DD . . . D	Atypical
	Q . . . . DG . . . G	Atypical
	GGNEGRGYGGRGEGGAEDDRRPLHPERVNVFDY	I
	G . . . . . – – – – – . . . . – . . DRRP . . . GS . . E . . F	II
	R . . . . . GYGGR . . . . G . . DRRA . . . ER . . V . . Y	III
	G . . . . . GYGGR . . . . – . . GRGP . . . GS . . E . . F	Atypical
	R . . . . . GYGGR . . . . G . . DRRA . . . EH . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DRRA . . . ES . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DRRA . . . GS . . V . . Y	Atypical
198-230	G . . . . . – – – – – . . . .G . . DRRA . . . GS . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DRRA . . . ER . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DRRA . . . EH . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DGRA . . . ER . . V . . Y	Atypical
	G . . . . . GYRGR . . . . – . . DGRA . . . ER . . V . . Y	Atypical
	G . . . . . GYRGR . . . . G . . DRRP . . . GS . . V . . Y	Atypical
	G . . . . . GYGGR . . . . – . . DRRP . . . ER . . E . . Y	Atypical
	G . . . . . GDGGR . . . . G . . DRRP . . . ER . . E . . Y	Atypical

3.1.3 *GRA7* sequencing

GRA7 sequencing was only possible for 47 strains. The alignment of these 47 strains and the 3 reference strains showed 29 polymorphic positions at nucleotide level. Fourteen haplotypes were identified (Appendix 3 – Tables 1 and 4). In comparison with RH strain (haplotype 1), no deletions or insertions were found. Except for haplotype 1 that has a G, all the other haplotypes have a C at positions 505 and 523. Haplotypes 3, 4, 7, 8, 11, 12 and 13 are characterized by the following nucleotide substitutions: a G at position 340, a C at position 367, a T at position 550, a A at position 577, a T at position 605, a C at position 619, a A at position 625, a A at position 629, a C at position 683, a C at position 690 and a A at position 715. Twenty strains belong to haplotypes 1, 2 or 3 for *GRA7*. Haplotype 1 was found in 13 strains, mostly from South America or Africa: strain RH (type I reference strain), strain TgCkCo24 (variant of I), strain CRL-2004-MOT (III/I genotype) and in 10 atypical strains. Four out of six strains from Africa were haplotype 1. Haplotype 2 was only found for BEVERLEY (type II reference strain), for GANGI (variant of II) and for IPB-2003-COG (II/III genotype). Haplotype 3 was found in NED (type III reference strain), ENVL-2002-MAC (variant of III) and for two atypical strains from Brazil. Haplotype 9, 10 and 14 were exclusive from French Guiana strains. Haplotype 4 is the most frequent among strains from Brazil, being shared by 11 of the 23 studied strains. Haplotype 6 was found in one strain from Reunion Island, one strain from French Guiana and two Brazilian strains. Haplotype 5 was found in two strains from Brazil. Haplotypes 7, 11 and 12 were found in three strains from South America (Appendix 3 - Table 1).

3.1.4 Selection of polymorphic peptides from GRA7 antigen

GRA7 locus codifies a protein of 236 aa. At amino acid level (Appendix 3 - Table 5), only 10 different sequences were found. Amino acid sequences were identical for haplotypes 3 and 11, for haplotypes 4, 7 and 12, for haplotypes 9 and 10.

The most polymorphic region at GRA7 protein sequence comprises the aa 170 to 182. Six different peptides can be selected at this region to differentiate strains (Table 10). Peptides [R–D–T–G–G] and [T–E–T–G–S] are exclusive of GRA7 haplotype 1 and 2 respectively. Most African strains (5 out of 6) shared the peptide [R–D–T–G–G] characteristic of haplotype 1. Peptide [T–D–S–G–S] is shared by haplotypes 3, 4, 7, 8, 11, 12 and 13. This peptide is

found in 15 out of the 23 Brazilian strains. Peptides [A–E–T–S–N], [A–D–T–S–N] and [T–E–T–G–G] are exclusive of non-archetypal strains. Peptide [A–E–T–S–N], shared by strains belonging to haplotypes 6, 9 and 10 is found in ten strains. Except TOU-2002-ALI (haplotype 6) strain isolated in Reunion Island in the Indian Ocean, the remaining nine strains are atypical and from South America. This peptide is found in seven from nine strains from French Guiana with human origin. It differs from peptide [A–D–T–S–N] found in 2 Brazilian strains (TgCkBr3 and TgCkBr22) by a single aa.

The regions between position 106-120, 199-222 and 220-236 are less polymorphic (Table 10 and Appendix 3- Table 5). In these regions most of the atypical strains shared the polymorphisms with GRA7 type I, II or III. The region between positions 106 and 120 allows differentiating 12 of the 23 Brazilian strains (from haplotypes 4, 7, 8 and 12). Only two different peptides ([M–LT–L–Q] and [L–IK–P–H]) can be selected from the region between aa 199 and 222. These peptides differ by five aa and allows differentiating GRA7 type I and II from type III respectively. Peptide [M–LT–L–Q] is found in the six African strains and in nine out of ten strains from French Guiana. Three different peptides are found between aa 220 and 236 ([L–Q–E–G], [L–Q–K–G] and [P–H–E–R]) that allow differentiating GRA7 type I, II and III. Peptides [L-Q-E-G] and [P-H-E-R] are also found in atypical strains. Peptide [L-Q-E-G] is found in five of the six African strains and in nine out of ten strains from French Guiana.

Table 10: Polymorphic peptides from *GRA7* marker. Periods (.) indicate identical amino acid.

	Polymorphic peptide	Strain type
106-120	RSDAEVTDDNIYEEH	I, II, Atypical
	G . . . . . H . . . . N	III, Atypical
	G . . . . . H . . . . H	Atypical
170-182	RGDEPLTTGQNVG	I
	T . E . . . T . G . . . S	II
	T . D . . . S . G . . . S	III, Atypical
	A . E . . . T . S . . . N	Atypical
	A . D . . . T . S . . . N	Atypical
	T . E . . . T . G . . . G	Atypical
199-222	MGLTRTYRHFSPRKNRSRQPALEQ	I, II, Atypical
	L . IK . . . . . P . H	III, Atypical
220-236	LEQEVPESGEDGEDARQ	I, Atypical
	L . Q . . . . . K . G	II
	P . H . . . . . E . R.	III, Atypical

3.1.5 *GRA8* sequencing

Only 19 strains were sequenced. The alignment of these 19 strains with a reference strain (RH) showed 18 polymorphic positions at nucleotide level (Appendix 3 – Tables 1 and 6). *GRA8* locus was not amplified in the studied type II strains. As consequence, *GRA8* was

not sequenced in type II strains. Only five different haplotypes were found. Ten out of 19 strains belong, together with strain RH (type I), to haplotype 1. NED (type III), four strains from French Guiana (GUY-2001-DOS, GUY-2002-MAT, GUY-2004-LAB and GUY-2004-TER), three strains from Brazil (MAR-2000-HOU, TgCkBr3 and TgCkBr9) and two African strains (WIK and PSP-2003-KOM) belong to haplotype 1. Haplotype 3, which includes the strains GUY-2002-KOE and GUY-2004-AKO, differed from haplotype 1 by a single polymorphism at position 414. Haplotype 4 was exclusively found in GUY-2004-JAG. Haplotype 5 was exclusively found in chickens from Brazil (TgCkBr1, TgCkBr6 and TgCkBr18). This haplotype differed from haplotype 1, by thirteen polymorphisms. Haplotype 2 was found in the strains RMS-1994-LEF, TgCkAr23 and TgCkBr4. Ten polymorphic positions and one deletion of 3bp was found in this haplotype.

3.1.6 Selection of polymorphic peptides from GRA8 antigen

GRA8 locus codes for a protein of 267 aa. At amino acid level, five sequences were found with 12 polymorphic positions (Appendix 3 – Table 7). A deletion of 1 amino acid at position 196 was found in strains from haplotype 2. This is not a very polymorphic locus. Most of the sequenced strains shared the same sequence of amino acids, also shared by strains type I and III, which makes this antigen not very useful for selection of possible peptides to be used in serotyping.

3.1.7 Immunogenic B-cell epitope selection

GRA6 and GRA7 aa sequence of type I, II and III reference strains were analysed for their hydrophobic, hydrophilic and antigenic character in order to select potential antigenic sites. Hydrophilic regions are likely exposed on the surface of a protein and may possibly be antigenic. Hydrophobic regions usually correspond to non-antigenic membrane-spanning domains. GRA8 sequences were not analysed for epitopes selection because this antigen is not very polymorphic

Analysis of GRA6 sequences from strains type I, II and III for hydrophilicity and antigenicity characteristics revealed two potential antigenic regions. Sequence between positions 138 and 154, was considered hydrophilic and antigenic according to the Hoop and Woods hydrophilicity scale and the Welling antigenicity scale. C-terminal region between

position 215 and 226 was also considered hydrophilic and antigenic. Sequence between position 198 and 214, was considered hydrophilic according the Hoop and Woods scale. These regions were not considered hydrophobic according to the parameters established by Eisenberg (Table 11).

Analysis of GRA7 sequences from strains type I, II and III revealed four potential antigenic regions. Sequence between positions 72 and 141, was considered hydrophilic according to the Hoop and Woods scale. Welling antigenicity scale selected some regions in this sequence as possible antigenic ones. Sequence between positions 159 and 175, was considered hydrophilic according to the Hoop and Woods scale. Welling scale and the Bioinformatics software selected the region between positions 152 and 163 as possibly antigenic. C-terminal region between positions 204 and 233 was considered hydrophilic and potentially antigenic. Region between positions 178 and 200 was considered antigenic by bioinformatics, but it was also considered hydrophobic according the Eisenberg scale (Table 11). Hydrophobic regions are usually not at membrane surface and are expected to be less antigenic.

Table 11: Antigenic prediction for GRA6 and GRA7 proteins. Sequences referred are specific for type I strains. Polymorphic aa are placed in red.

Loci	Position	Sequence	Parameters
GRA6	138-154	PQAKVPSKRTQKRHRLI	Antigenic/Hydrophilic
	198-214	GGNEGRGYGGRGEGGAE	Hydrophilic
	215-226	DDRRPLHPERVN	Antigenic/Hydrophilic
GRA7	72-141	SMDKASVESQLPRREPLETEPDEQEEVHFRKRGVRSD AEVTDDNIYEEHTDRKVVPRKSEGKRSFKDLLK	Antigenic/Hydrophilic
	152-163	ASYFAADRLVPE	Antigenic
	159-175	RLVPELTEEQQRGDEPL	Hydrophilic
	178-200	GQNVGTVLGFAAL AAAAAFLGMG	Antigenic/Hydrophobic
	204-233	TYRHFSPRKNRSRQPALEQEVPESGEDGED	Antigenic/Hydrophilic

3.1.8 Polymorphic peptides definition

Peptides were selected from regions that were at the same time polymorphic and immunogenic. The regions selected as immunogenic were crossed with the polymorphic positions of GRA6 and GRA7 antigens. Seven peptides were defined (Table 12). For GRA6, from the sequences selected as potentially immunogenic, only the C-terminal region was also polymorphic. Between aa 198 and 226 specific polymorphisms allowed differentiating atypical strains from the archetypal lineages. This polymorphic and potentially immunogenic sequence was selected to define peptides specific for both archetypal and atypical strains. The two peptides derived from GRA6 antigen and specific for the archetypal lineages were based on the peptides defined by Kong and colleagues (2003). The two GRA6 peptides specific for the non archetypal strains were selected from the most representative haplotypes found for Africa and South America. Haplotype 11 is shared by four out of six African strains, which justifies the selection of peptide Af6. Peptide Am6 was selected from haplotype 7. For South American strains, the most representative haplotypes were haplotypes 7 and 10. But, the number of polymorphic aa between haplotypes 7 and 11 was higher than between haplotypes 10 and 11. Consequently a better differentiation of atypical strains was expected with one peptide derived from haplotype 7 than from haplotype 10. For GRA7, the sequences selected by both antigenic and hydrophilic scales were between aa 72 and 141 and between aa 204-233. The sequence between aa 72 and 141, only has three polymorphic positions, which is a low rate of polymorphisms considering the size of the sequence. Polymorphic positions in the sequence between aa 204 and 233, allowed differentiating type I from type III strains. Based on this, peptides specific for these two lineages were selected between aa 220 and 236. Sequence between aa 170 and 182 in GRA7 antigen is specific for the atypical strains of French Guiana. This sequence partially includes two regions selected as potentially immunogenic, which justifies the selection of peptide Am7.

For sequences with less than 20 amino acids (all but Am6 and Af6), the selected region was repeated three times in one single peptide to enhance its recognition in immunoenzymatic assay.

Table 12: Amino acid sequence of GRA6 and GRA7 synthetic peptides. The polymorphic aa are placed in red.

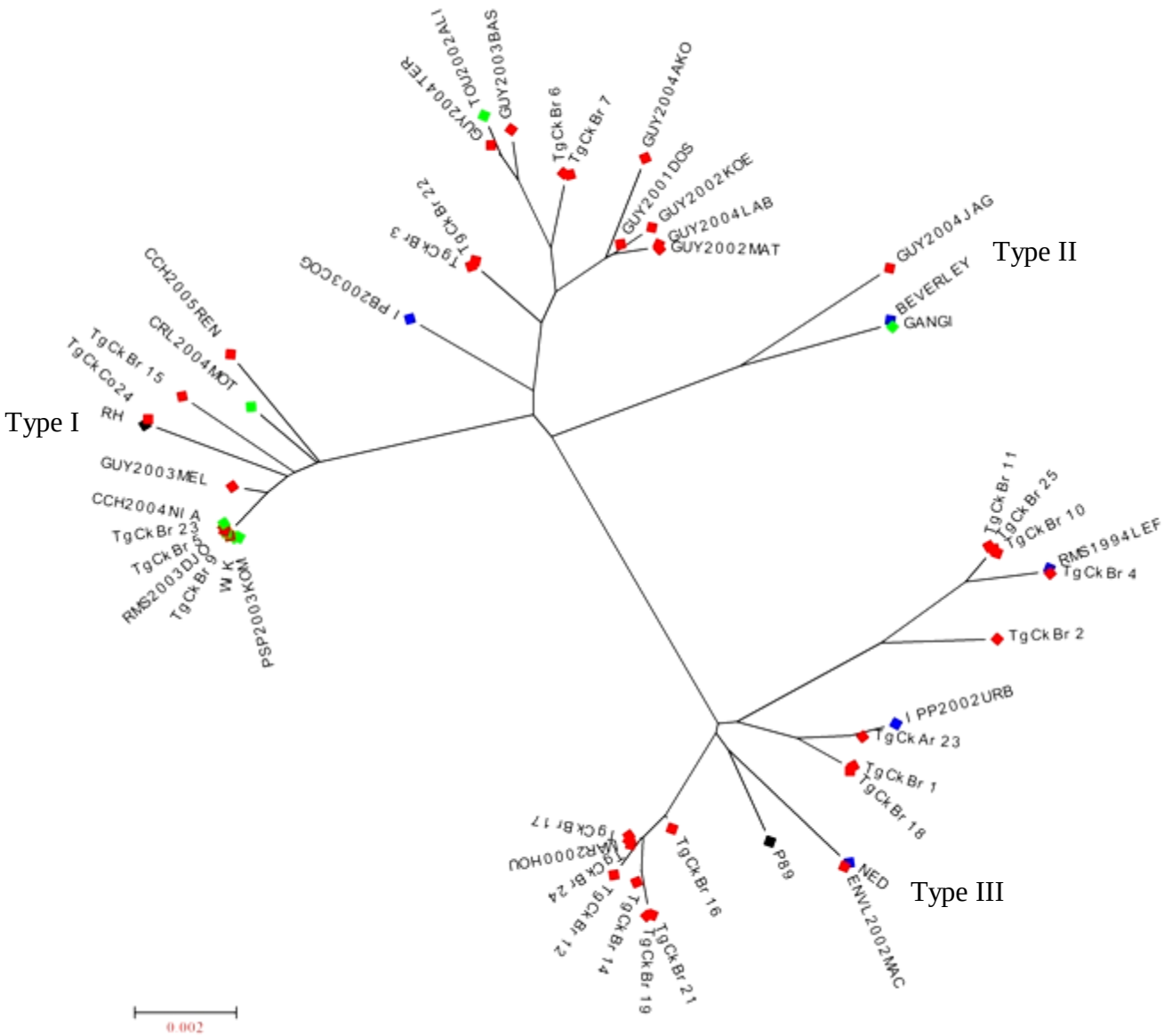
Peptide	Loci	aa position	Strain type	Amino acid sequence
GRA6II	*GRA6*	220-230	II	LHPGSVNEFDFLHPGSVNEFDFLHPGSVNEFDF
GRA6I/III	*GRA6*	220-230	I and III	LHPERVNVFDYLHPERVNVFDYLHPERVNVFDY
GRA7I	*GRA7*	220-236	I	LEQEVPESGEDGEDARQLEQEVPESGEDGEDARQLEQEVPESGEDGEDARQ
GRA7III	*GRA7*	220-236	III	PEHEVPESGEDREDARQPEHEVPESGEDREDARQPEHEVPESGEDREDARQ
Am6	*GRA6*	198-230	Atypical (haplotype 7)	GGNEGRGEGGGEDDRRALHPGSVNVFDY
Af6	*GRA6*	198-230	Atypical (haplotype 11)	GGNEGRGYRGRGEGGGEDDGRALHPERVNVFDY
Am7	*GRA7*	170-182	Atypical (haplotypes 6, 9 and 10)	AGEEPLTTSQNVNAGEEPLTTSQNVNAGEEPLTTSQNVN

3.1.9 Phylogenetic analysis

Phylogenetic distance (Neighbor Joining method) between the studied strains was analysed, based on the nucleotide sequences for *GRA6* and *GRA7* loci. Four distinct branches were defined (Figure 7). The three clonal lineages (I, II and III) are placed in three distinct branches. Type I strains are clustered together with five out of six strains from Africa and with seven strains from South America or Caribbean Islands. Type II strains and the atypical strain GUY-2004-JAG derived from the same ancestor and form a separated cluster. Strains type III are clustered with fifteen out of twenty-three Brazilian strains. All African and French Guianan strains are more closely related with strains type I and III. Seven out of nine strains from French Guiana are clustered together in a branch derived from a ancestor common to type I strains.

Figure 7: Phylogenetic tree determined by analysis of the sequences of *GRA6* and *GRA7*. These markers were studied by distance analyses method (Neighbor Joining). A) Radial tree B) Circular tree.

A)



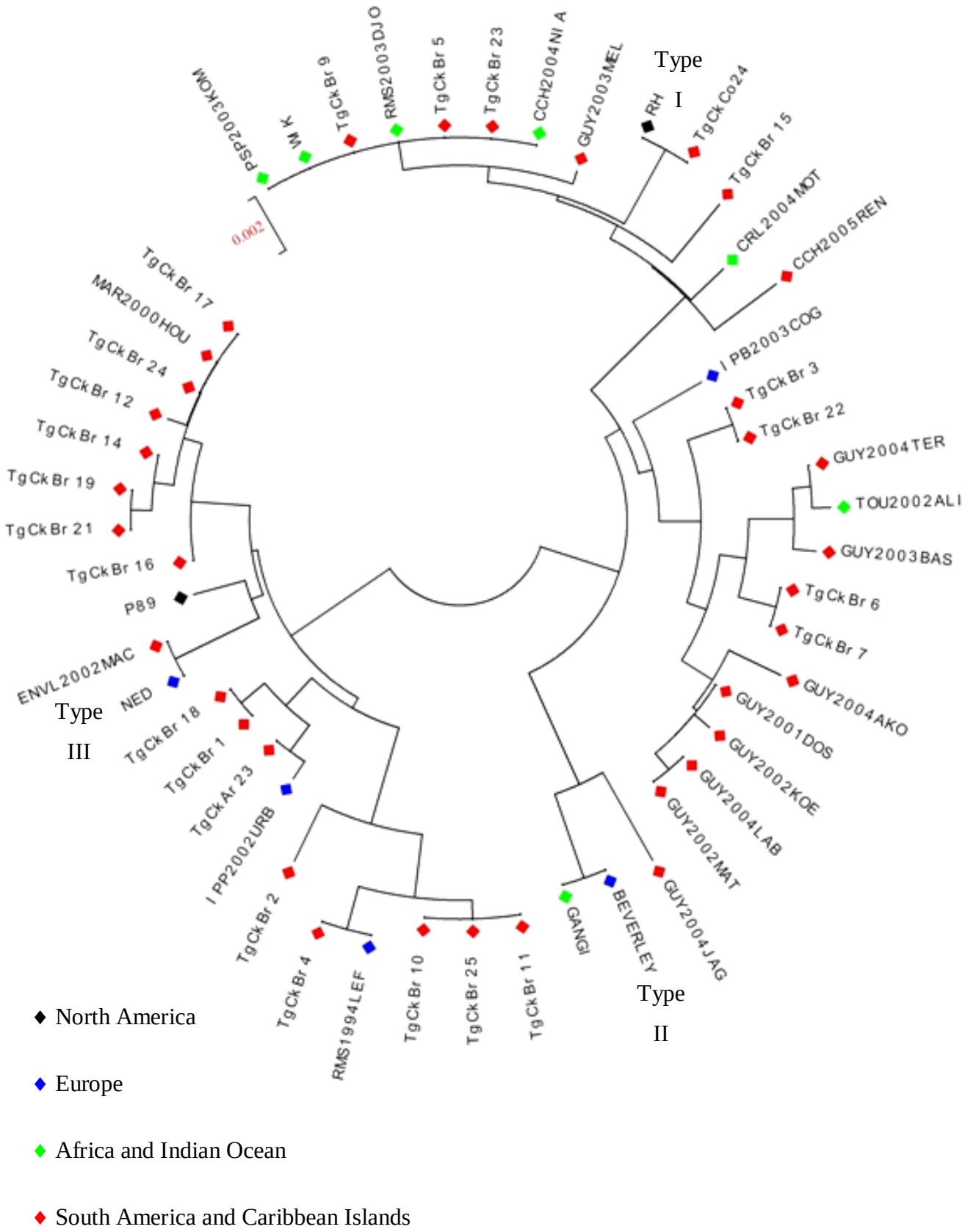
♦ North America

♦ Europe

♦ Africa and Indian Ocean

♦ South America and Caribbean Islands

B)


Type I
Type III
Type II
0.002
RH
TgCkCo24
TgCkBr15
CRL2004MOT
CCH2005REN
IPB2003COG
TgCkBr3
TgCkBr22
GUY2004TER
TOU2002ALI
GUY2003BAS
TgCkBr6
TgCkBr7
GUY2004AKO
GUY2001DOS
GUY2002KOE
GUY2004LAB
GUY2002MAT
GUY2004JAG
BEVERLEY
GANGI
TgCkBr11
TgCkBr25
TgCkBr10
RMS1994LEF
TgCkBr4
TgCkBr2
IPP2002URB
TgCkAr23
TgCkBr1
TgCkBr18
NED
ENVL2002MAC
P89
TgCkBr16
TgCkBr21
TgCkBr19
TgCkBr14
TgCkBr12
TgCkBr24
MAR2000HOU
TgCkBr17
PSP2003KOM
KWK
TgCkBr9
RMS2003DJO
TgCkBr5
TgCkBr23
CCH2004NIA
GUY2003MEL
North America
Europe
Africa and Indian Ocean
South America and Caribbean Islands

3.2 Genetic characterization of *Toxoplasma gondii* infections by serotyping

3.2.1 Serotype definition

Different reactivity profiles were found for the seven peptides. Based on the different profiles, different serotypes were defined (Table 13). Serum samples were considered positive when OD index was equal or above the cut-off defined for each peptide.

Table 13: Description of the different serotypes

Serotype	**Reactive peptides**
I	GRA7I GRA7I-GRA6I/III
II	GRA6II
III	GRA7III GRA7III-GRA6I/III
GRA6I/III	GRA6I/III
Not determined (ND)	exclusive reaction with the peptides specific for the atypical strains
Non reactive (NR)	OD index below cut-off values for all peptides

	Serotype		Reactive peptides
Cross-reaction (CR) or mixed profile	I	I/III	GRA7I-GRA7III GRA7I-GRA7III-GRA6I/III
		II/II	GRA6II-GRA7III GRA6II-GRA7III-GRA6I/III
	III	II/I/	GRA6II-GRA6I/III GRA6II-GRA7I-GRA7III GRA6II-GRA7I-GRA7III-GRA6I/III
		II/I	GRA6II-GRA7I GRA6II-GRA7I-GRA6I/III

3.2.2 Experimentally infected or immunized animals

3.2.2.1 Reactivity against total Ig

Thirty serum samples from *T. gondii* seronegative mice were used to establish the cut-off values for the different peptides. All seven peptides had a high specificity. Peptides GRA6I/III, GRA7III, Am6 and Am7 did not react with any negative serum sample. Peptides GRA6II and GRA7I reacted with one negative serum each. Peptide Af6 reacted with two negative serum samples.

It would be expected that serum samples from mice inoculated with type I and III strains reacted with peptide GRA6I/III specific for type I and III strains (Figure 8 and Appendix 5 – Table 1).

- Eight out of the nine serum samples from mice infected with type III strains recognized this peptide. On the contrary, GRA6I/III peptide was not recognized by any serum sample from mice immunized with type I strain antigen.
- Type II strains differed from this peptide (GRA6I/III) by 4 aa. Only one of the 27 serum samples from mice infected with type II strains reacted with peptide GRA6I/III.
- The strains MAR-2000-HOU and GUY-2004-AKO have only one aa of difference with peptide GRA6I/III. Serum samples from mice infected with MAR-2000-HOU did not recognize this peptide, but three out of the four serum samples from mice infected with GUY-2004-AKO reacted with peptide GRA6I/III.
- Strain TgCkAr23 differed by two aa from peptide GRA6I/III. Serum samples from mice infected with this strain did not recognize this peptide.

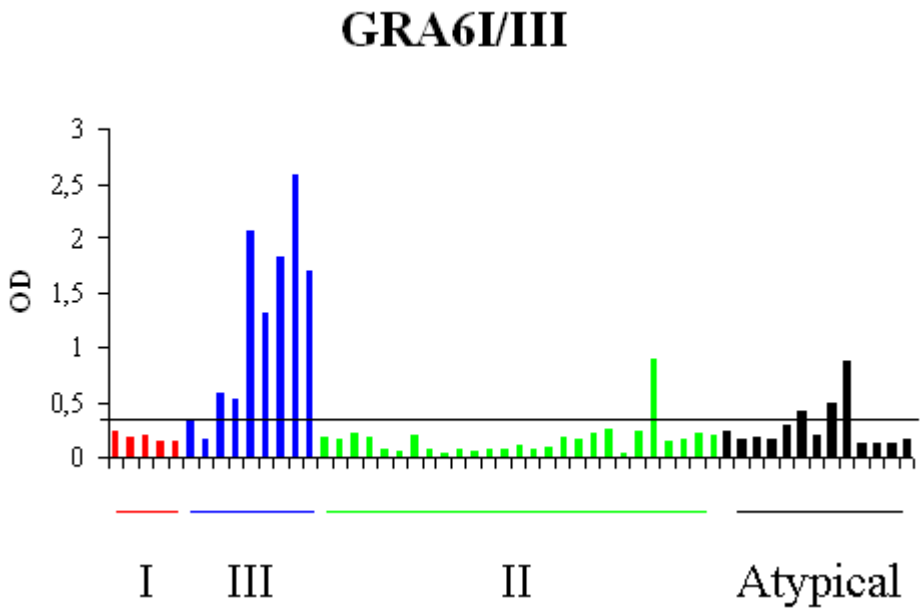


Figure 8: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide GRA6I/III. Cut-off is 0.327 (horizontal line).

Peptide GRA6II is specific for type II strains. However, not all serum samples from mice infected with type II strains recognized this peptide (Figure 9 and Appendix 5 – Table 1).

- Eight out of the 27 type II sera did not recognized peptide GRA6II.
- This peptide differs from type I, III, MAR-2000-HOU and GUY-2004-AKO strains by four aa and from TgCkAr23 by two aa. Peptide GRA6II was not recognized by serum samples from any mice infected with these strains.

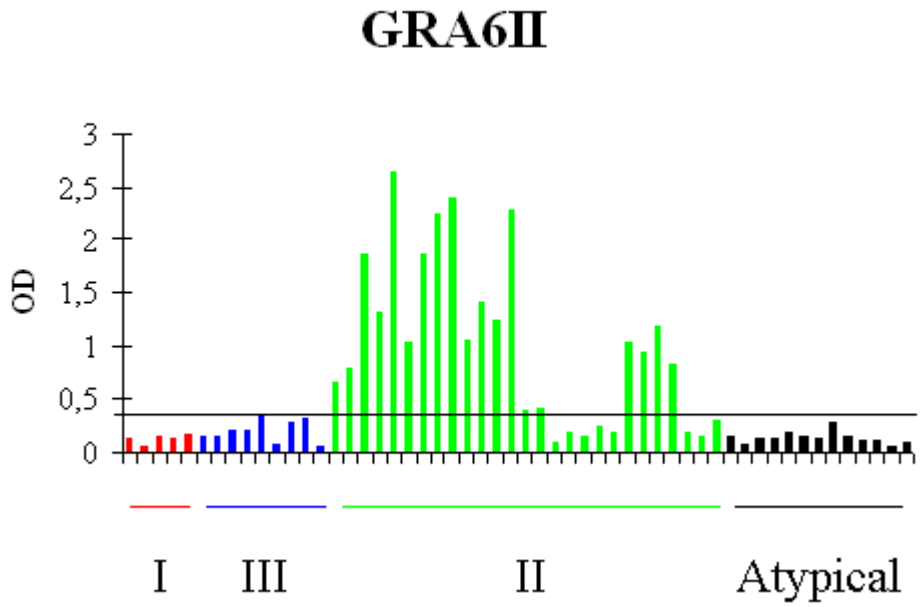


Figure 9: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide GRA6II. Cut-off is 0.369 (horizontal line).

Peptide GRA7I, specific for type I strains did not recognize any serum sample from mice immunized with lysate of the RH strain (type I) or infected by the strain GUY-2004-AKO that shares for the C-terminal region the same polymorphisms with type I strains. This peptide cross-reacted with serum samples from one mouse infected with a type II strain (one different aa) and from one mouse infected with a type III strain (3 different aa) (Figure 10 and Appendix 5 – Table 1).

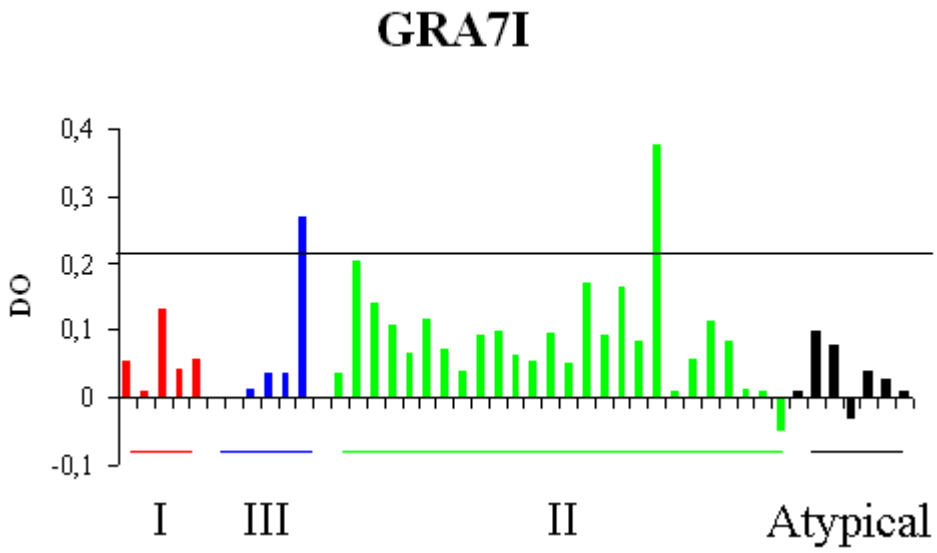


Figure 10: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide GRA7I. Cut-off is 0.213 (horizontal line).

Peptide GRA7III, specific for type III strains reacted with four out of seven serum samples from mice infected with type III strains. Mice infected with the atypical strains MAR-2000-HOU and TgCkAr23, which share with type III the same polymorphism for the GRA7 C-terminal region did not recognize this peptide. However, this peptide cross-react with two serum samples from mice infected with type II strains (4 different aa) and with one mouse infected with the atypical strain GUY-2004-AKO (3 different aa) (Figure 11 and Appendix 5 – Table 1).

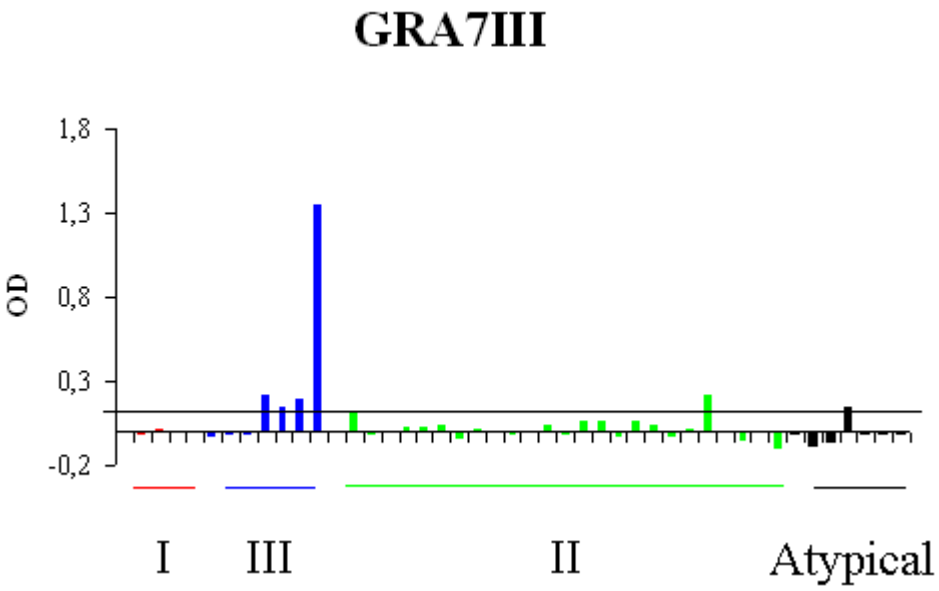


Figure 11: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide GRA7III. Cut-off is 0.113 (horizontal line).

Peptide Am6 differs by 4 aa and by 7 aa from type II and type III strains respectively. This peptide cross-reacted with serum samples from six mice infected with type III strains and with eighteen serum samples from mice infected with type II strains. Atypical strain GUY-2004-AKO differs by seven aa from peptide Am6. Four serum samples from mice infected with this strain cross-reacted with this peptide (Figure 12 and Appendix 5 – Table 1).

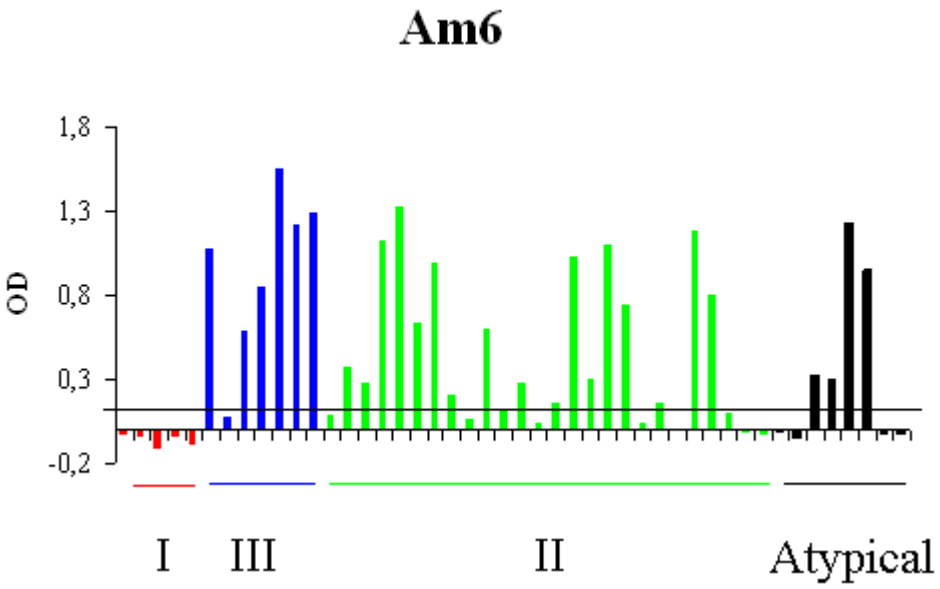


Figure 12: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide Am6. Cut-off is 0.109 (horizontal line).

Peptide Am7 was present in the strain GUY-2004-AKO. However, none of the mice infected with this strain reacted with this peptide. Four mice infected with type II strains and one mice infected with a type III strain recognized this peptide. Type II strains differ by 3 aa while type III strains differ by 5 aa from this peptide (Figure 13 and Appendix 5 – Table 1).

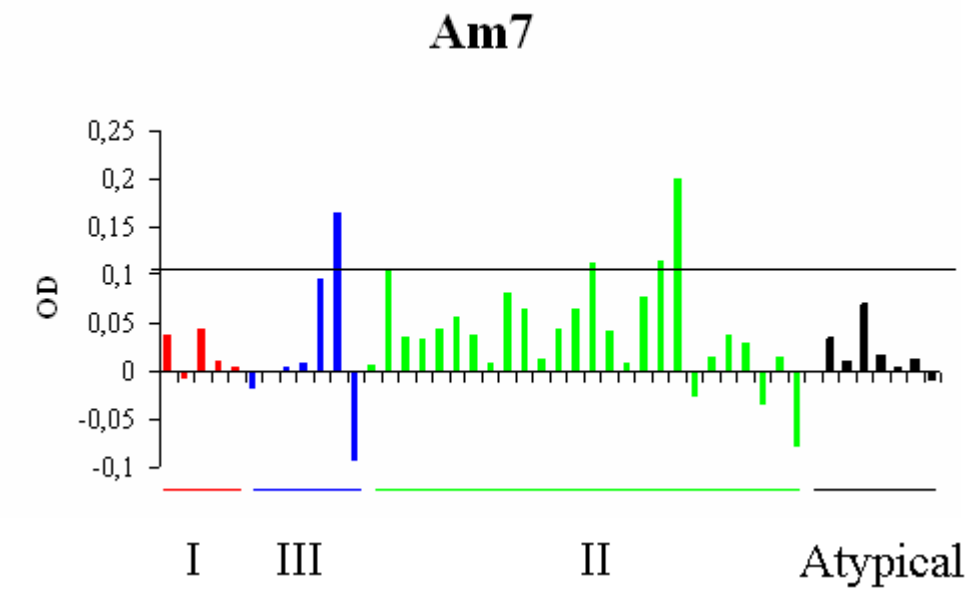


Figure 13: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide Am7. Cut-off is 0.105 (horizontal line).

Peptide Af6 differs from atypical strains GUY-2004-AKO and MAR-2000-HOU by two aa. One serum sample from one mouse infected with MAR-2000-HOU and four serum samples from mice infected with GUY-2004-AKO reacted with this peptide. Type II strains differ by 12 aa from this peptide. Nineteen serum samples from mice infected with type II strains recognized this peptide. Type III strains differ by three aa and seven serum samples from mice infected with these strains cross-reacted with peptide Af6 (Figure 14 and Appendix 5 – Table 1).

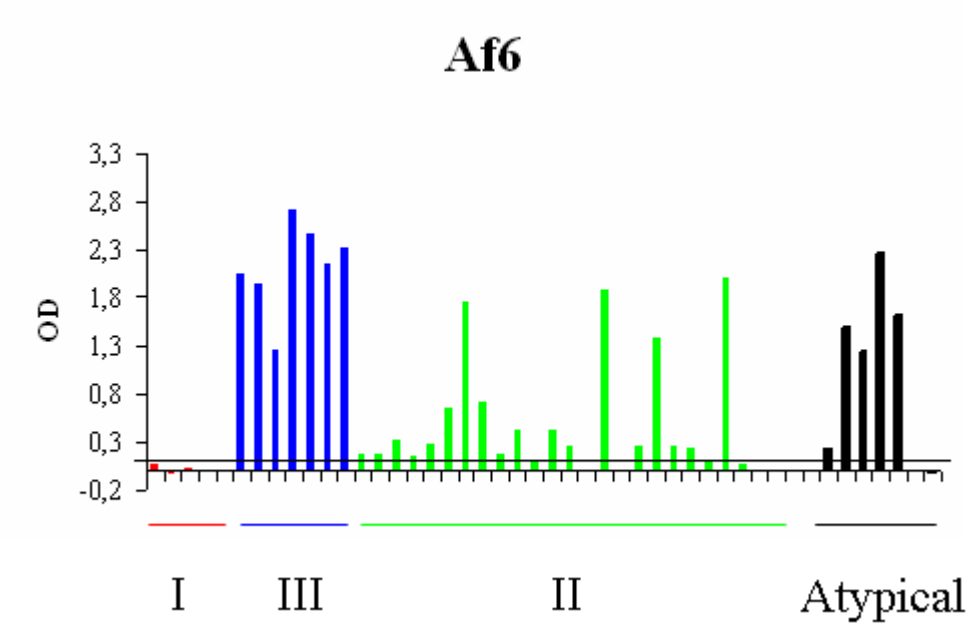


Figure 14: Pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide Af6. Cut-off is 0.128 (horizontal line).

The four peptides specific for the archetypal lineages showed a high specificity (from 90 to 100%) by the study of total Ig. Peptide GRA6II had a good sensitivity (70.4%). Peptide GRA6I/III showed a sensitivity of 88.9% for strains type III. Peptide GRA6I/III and GRA7I did not recognize strains type I. Peptide GRA7III showed a sensitivity of only 36.4%. Peptides specific for non-archetypal strains cross-reacted with serum samples from mice infected with strains type II and III. Peptides Am6 and Af6 showed a non-specific high reactivity rate, while peptide Am7 has a low reactivity rate.

3.2.2.2 Reactivity against IgG isotypes

Study of IgG isotypes, revealed that IgG2a was the most recognized (Appendix 5 – Table 2).

By the study of this isotype, serum samples from mice immunized with type I strain did not recognize the peptide GRA6I/III. Seven of the nine serum samples from mice infected with type III strains recognized this peptide. Only one serum samples from a mouse infected

with a type II strain reacted with peptide GRA6I/III. Serum samples from mice infected with atypical strains did not recognized this peptide, except two infected with the strain GUY-2004-AKO (Figure 15 and Appendix 5 – Table 2).

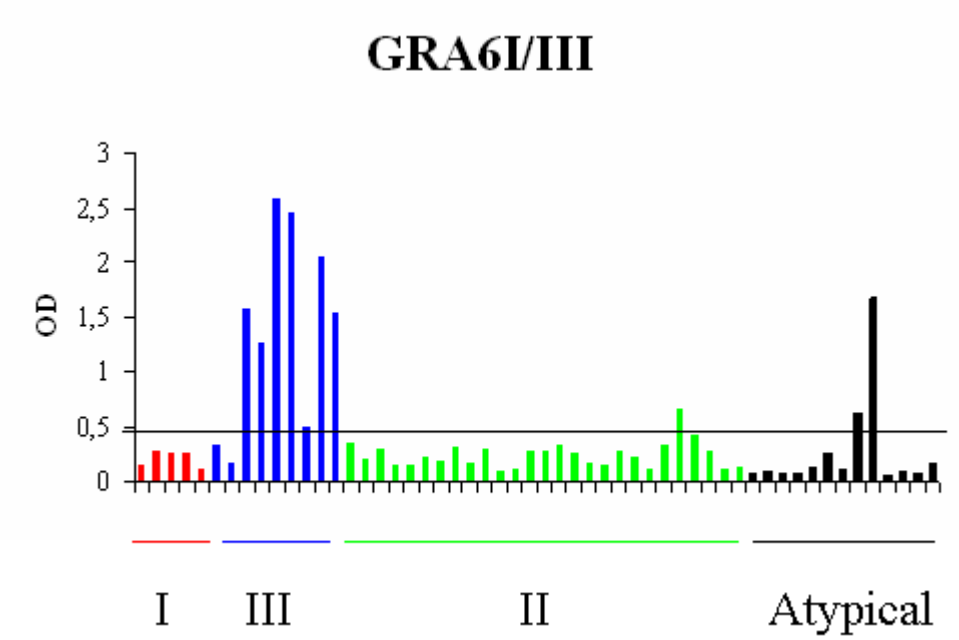


Figure 15: IgG2a pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate, against peptide GRA6I/III. Cut-off is 0.327 (horizontal line).

Peptide GRA6II was not recognized by 11 of the 27 serum samples from mice infected with type II strains by the study of IgG2a. Two of the type II sera (LGE-2006-BRO1 and LGE-2006-BRO2) recognized by this peptide were not recognized by the study of total IgG. However, five sera recognized by the study of total Ig were not recognized by the study of IgG2a. IgG2a antibodies of serum samples from one mouse infected with a type I strain, one infected with a type III strain, one infected with the strain MAR-2000-HOU and one infected with GUY-2004-AKO reacted with GRA6II peptide (Figure 16 and Appendix 5 – Table 2).

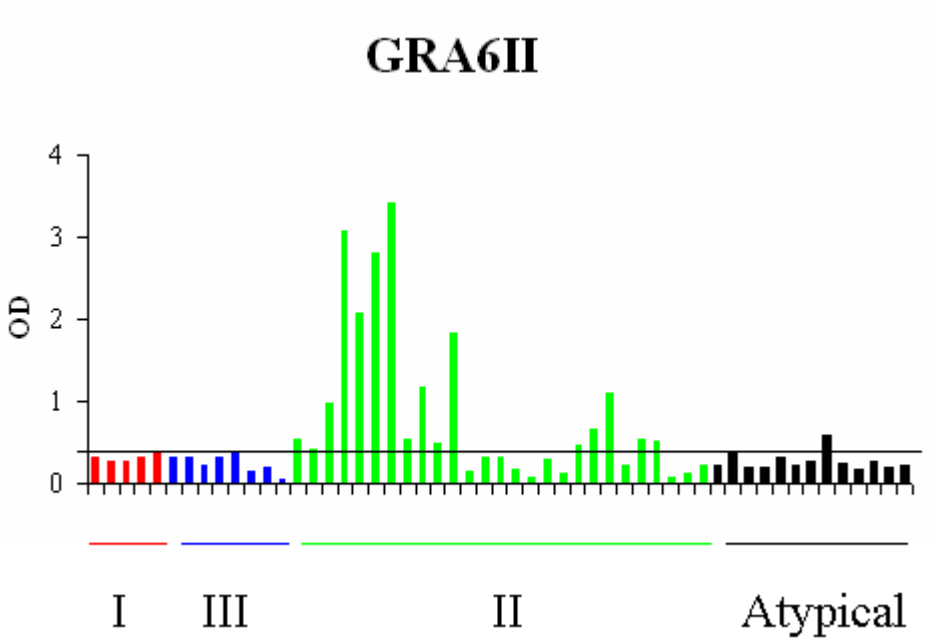


Figure 16: IgG2a pattern of reactivity of serum samples from mice infected with type II, III and atypical strains or immunized with type I lysate against peptide GRA6II. Cut-off is 0.369 (horizontal line).

Study of IgG2a (the most recognized isotype) showed a lower sensitivity for peptides GRA6II (59.3%) and GRA6I/III (50%) and a lower specificity for peptide GRA6II (84.6%).

3.2.3 Validation of *T. gondii* specific peptides for serotyping of naturally infected animals

Serotyping results from 11 chickens and 15 pigs are reported in tables 3 and 4 (Appendix 5).

From eight chickens infected with type II strains, five recognized the type II specific peptide GRA6II. From these five chickens, one also reacted with the peptide GRA6 I/III (GA167) and other reacted with the peptide GRA7 I (GA164). From three chickens infected with type III strains, only one (GA43) reacted with peptide GRA6 I/III. None of the chickens infected with type III strains reacted with peptide GRA7 III (Figures 17 to 20).

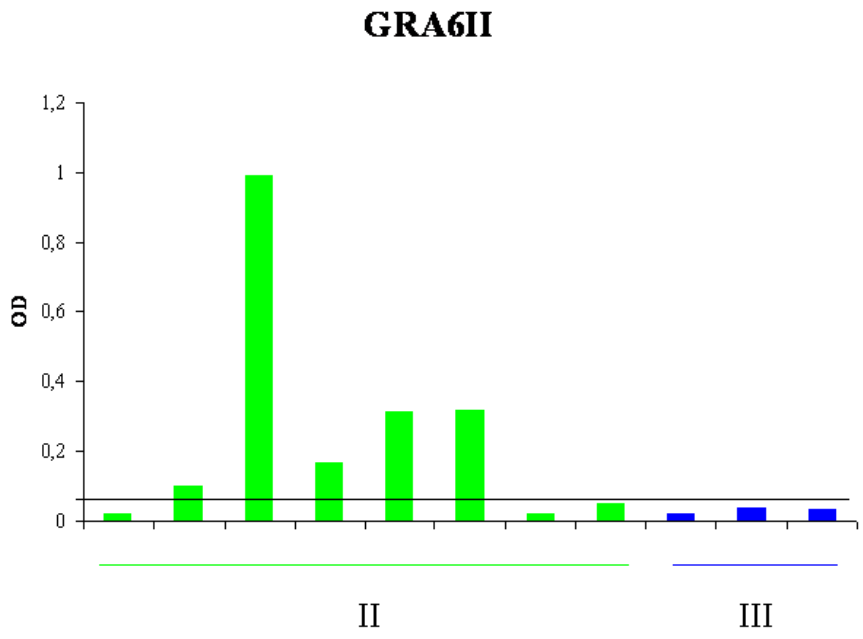


Figure 17: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide GRA6II. Cut-off is 0.058 (horizontal line).

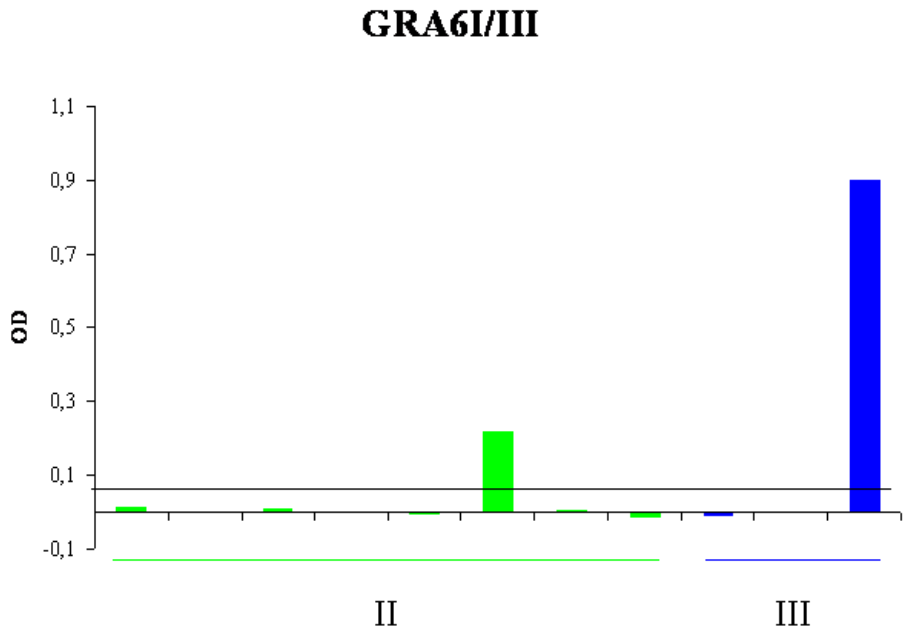


Figure 18: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide GRA6I/III. Cut-off is 0.051 (horizontal line).

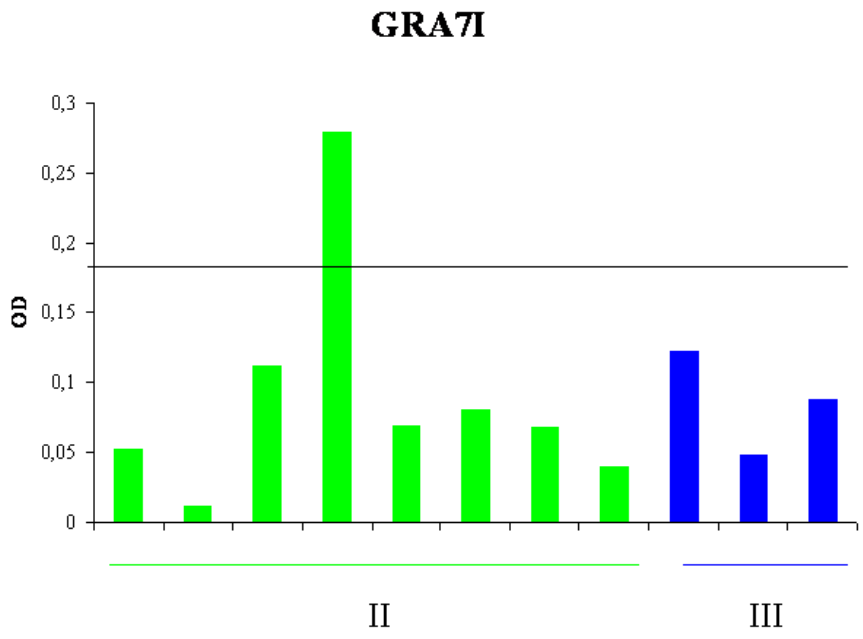


Figure 19: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide GRA7I. Cut-off is 0.180 (horizontal line).

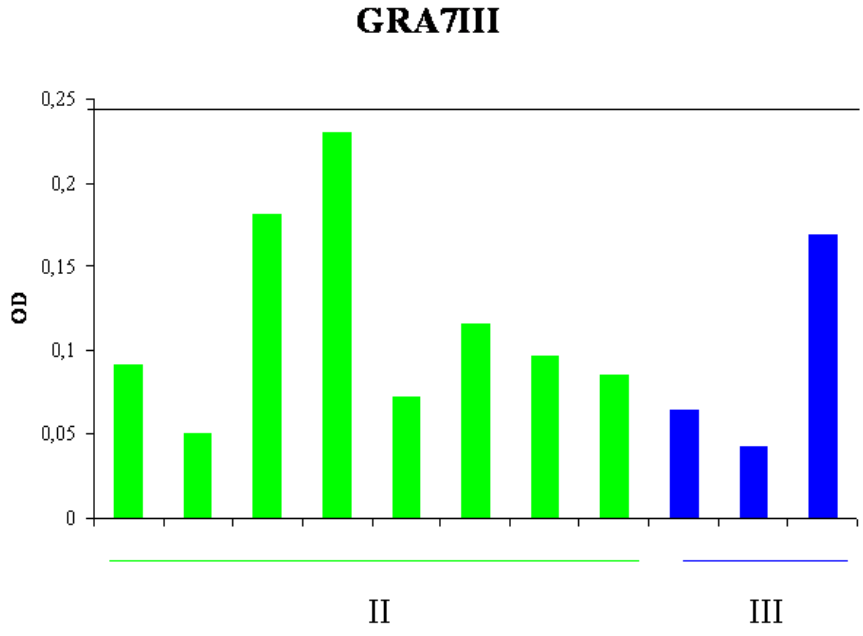


Figure 20: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide GRA7III. Cut-off is 0.244 (horizontal line).

From eleven pigs infected with type II strains, only two (PV227 and PV238) specifically recognized the peptide GRA6II. Three other pigs infected with type II strains had mixed reactivity profiles. Serum samples from PV272 and PV274 recognized the peptides GRA6II and GRA6I/III, and PV231 recognized the peptides GRA6 II, GRA6 I/III and GRA7III. One pig infected with a type II strain (PV232), specifically recognized the peptide GRA6I/III. Any of the four pigs infected with type III strains reacted with the specific peptides GRA6I/III and GRA7III (Figures 21 to 24).

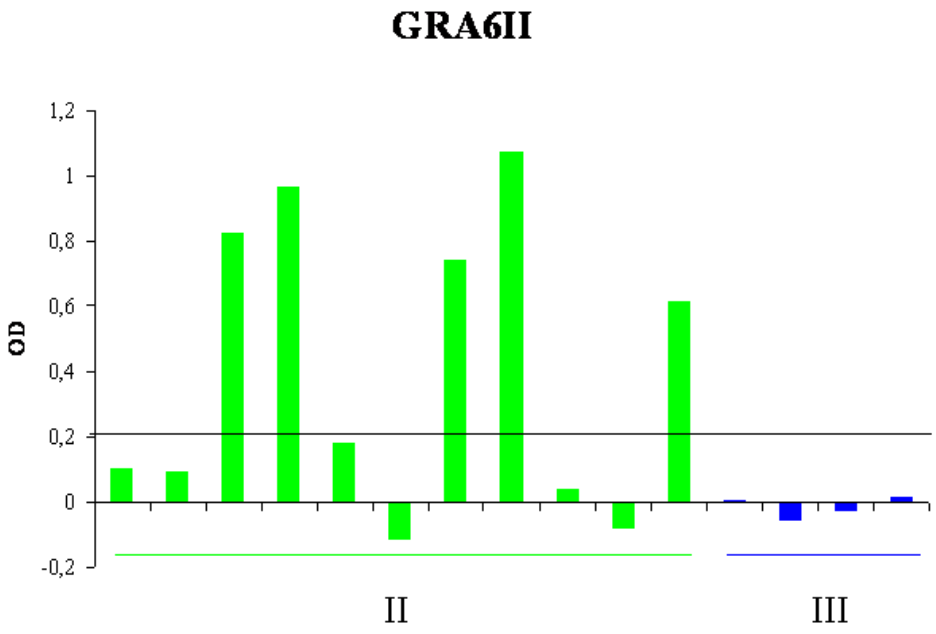


Figure 21: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide GRA6II. Cut-off is 0.212 (horizontal line).

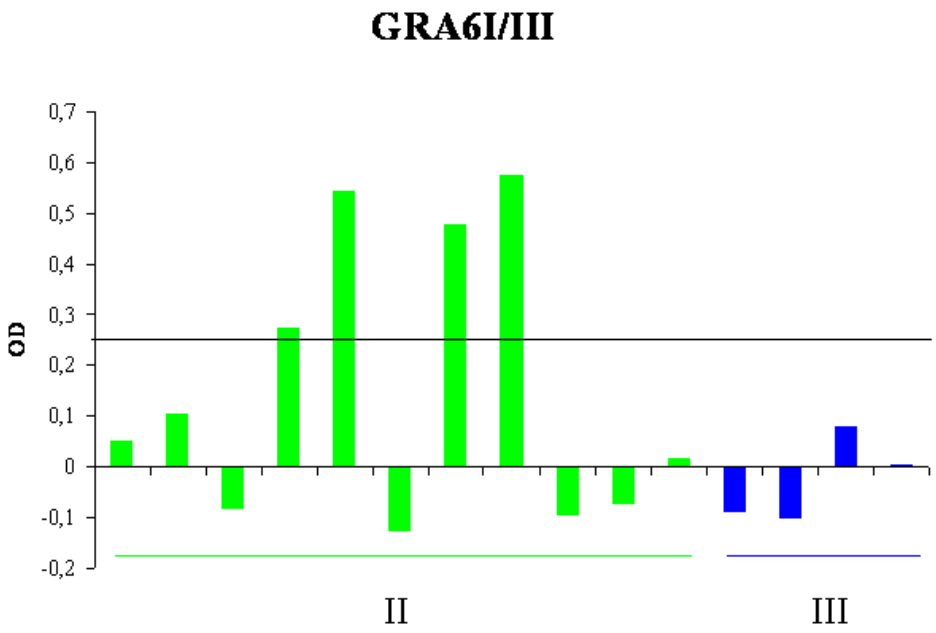


Figure 22: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide GRA6I/III. Cut-off is 0.249 (horizontal line).

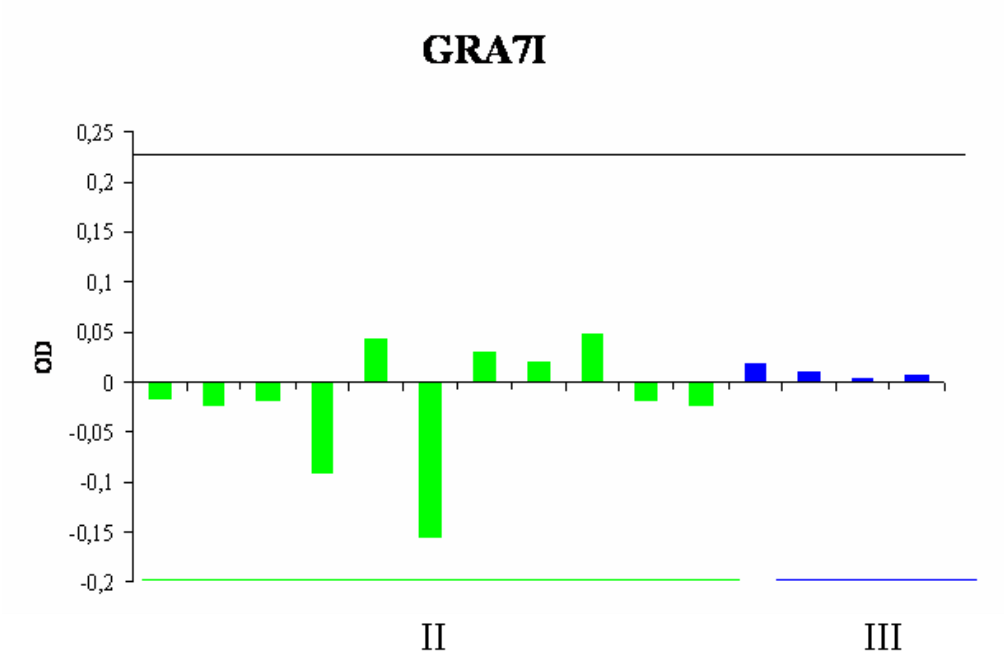


Figure 23: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide GRA7I. Cut-off is 0.231 (horizontal line).

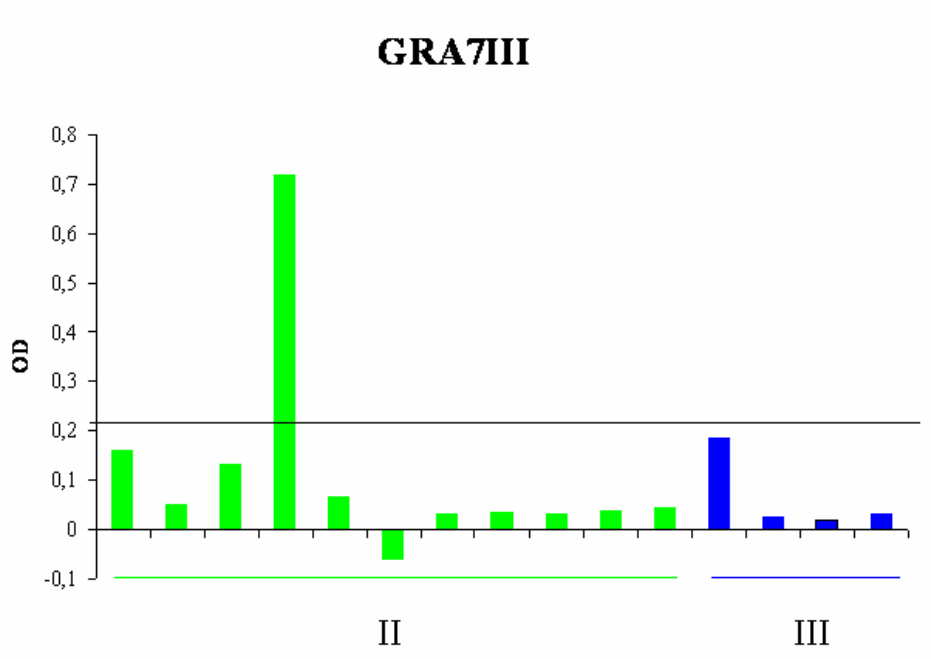


Figure 24: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide GRA7III. Cut-off is 0.228 (horizontal line).

Peptides specific for atypical strains also reacted with these sera (Appendix 5 – Tables 3 and 4). Peptide Am6 reacted with seven serum samples from chickens (five infected with type II strains and two infected with type III) (Figure 25) and three serum samples from pigs (two from pigs infected with type II strains (PV232 and PV274) and one infected with a type III strain (PV316)) (Figure 26).

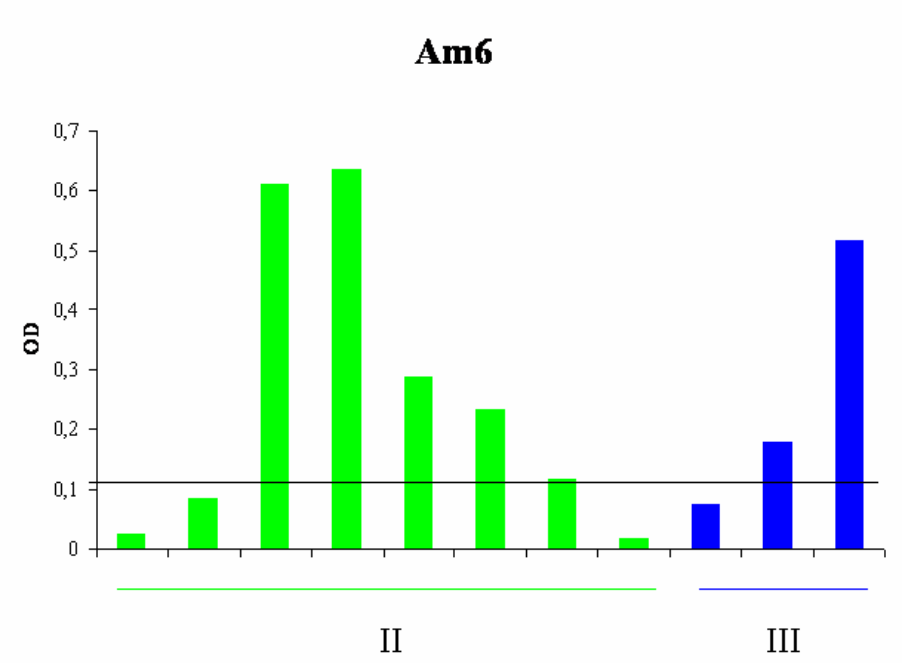


Figure 25: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide Am6. Cut-off is 0.115 (horizontal line).

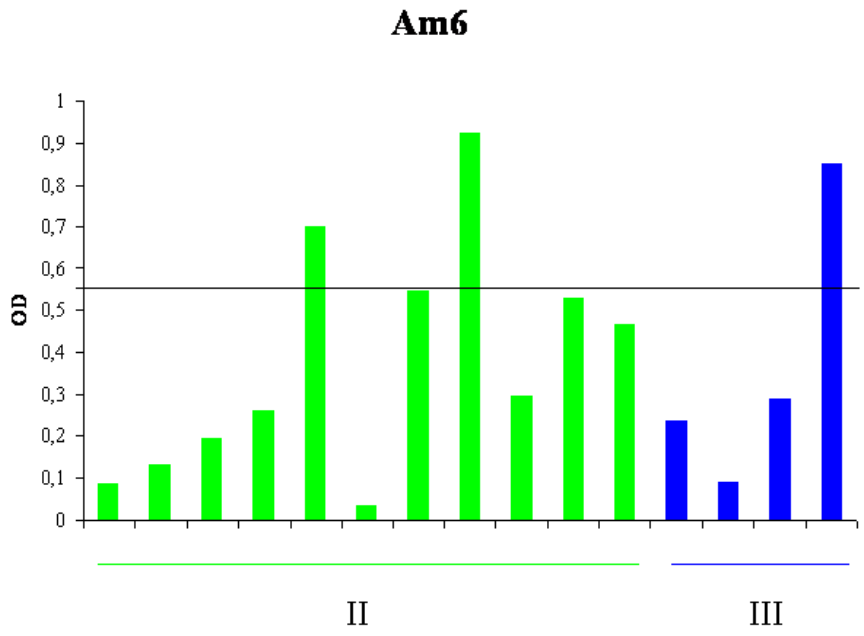


Figure 26: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide Am6. Cut-off is 0.549 (horizontal line).

No serum samples from pigs reacted with peptide Am7 and only one serum from a chicken (GA19) recognized this peptide (Figures 27 and 28). Peptide Af6 was also recognized by serum samples from pigs and chickens. Three serum samples from chickens reacted with this peptide (Figure 29): two from chickens infected with type II strains (GA163 and GA166) and one infected with a type III (GA43). Four sera from pigs infected with type II strains recognized this peptide (PV232, PV274, PV282 and PV302) (Figure 30).

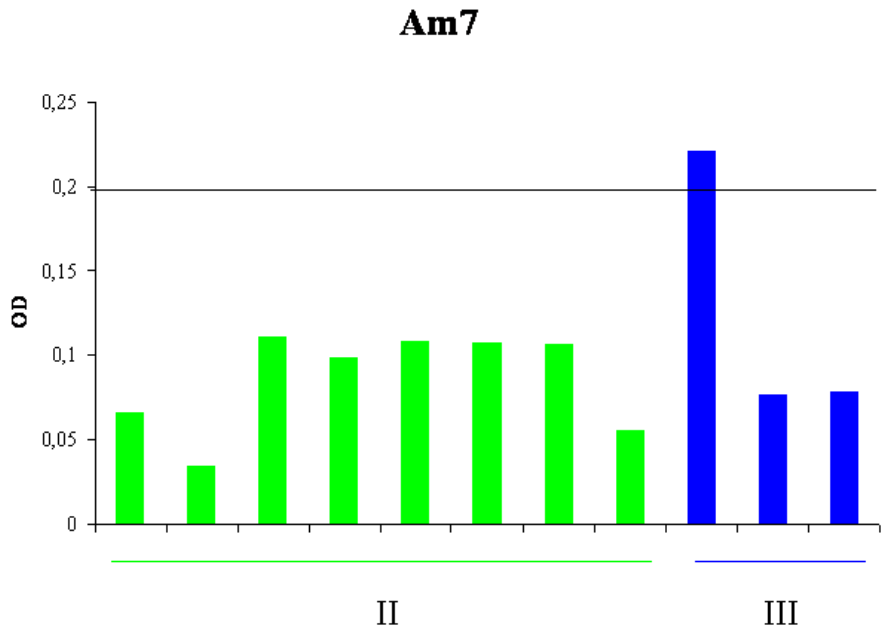


Figure 27: Pattern of reactivity of serum samples from chickens infected with type II and III strains against peptide Am7. Cut-off is 0.195 (horizontal line).

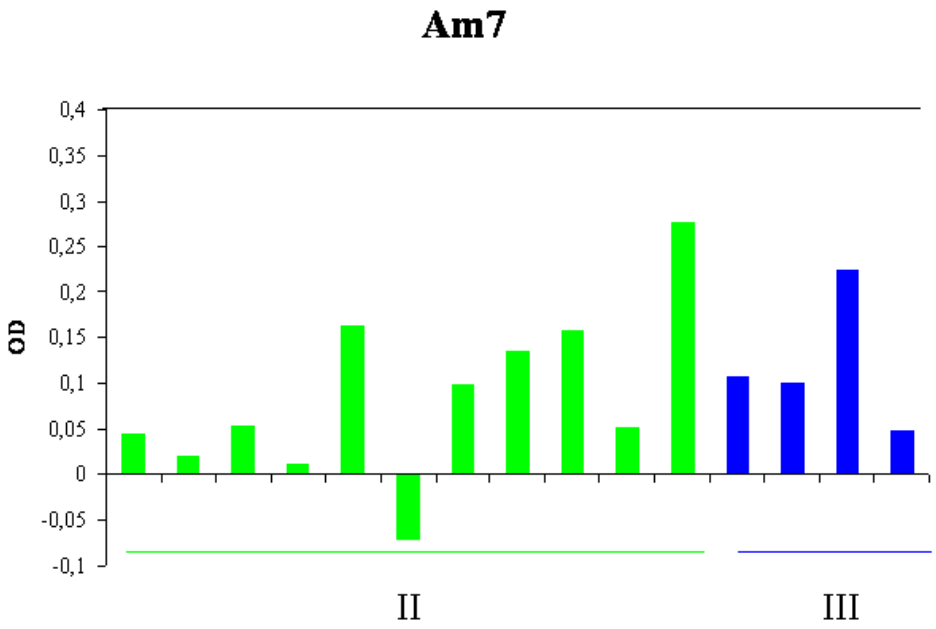


Figure 28: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide Am7. Cut-off is 0.403 (horizontal line).

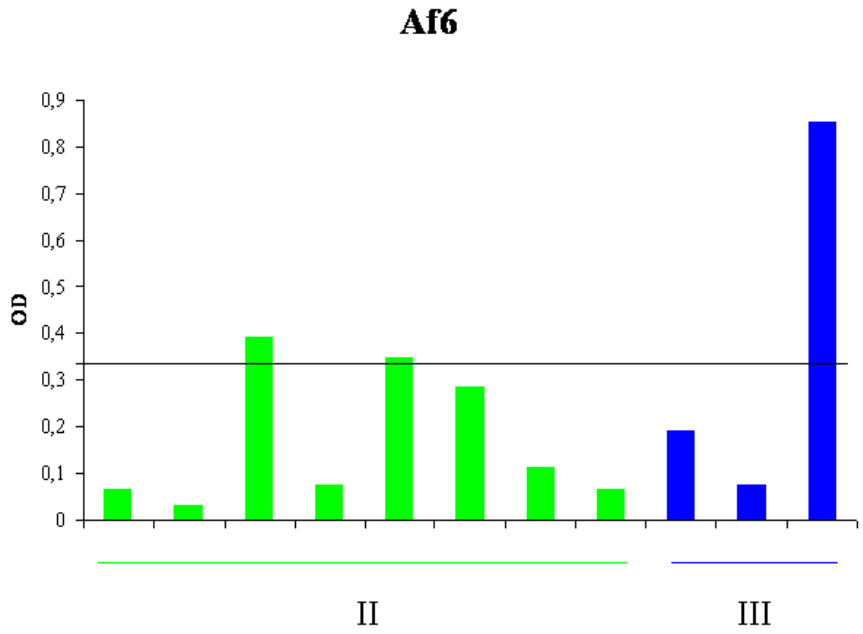


Figure 29: Pattern of reactivity of serum samples from chickens infected with strains type II and III against peptide Af6. Cut-off is 0.322 (horizontal line).

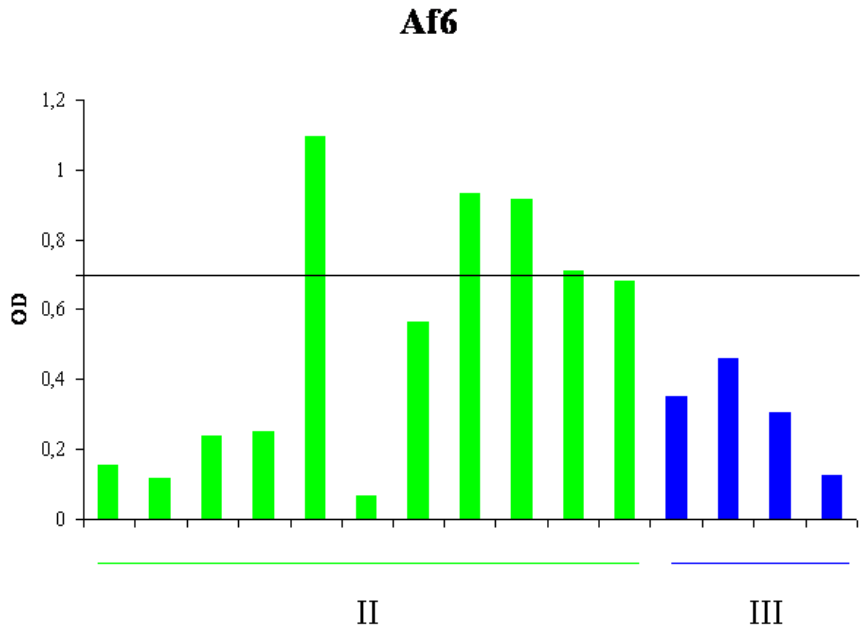


Figure 30: Pattern of reactivity of serum samples from pigs infected with type II and III strains against peptide Af6. Cut-off is 0.704 (horizontal line).

3.2.4 Prediction of *Toxoplasma gondii* serotypes in naturally infected animals from Portugal

Chickens, pigs and sheep positive for *T. gondii* from which no isolate was obtained were serotyped. Cut-off values for chickens and pigs were refered previously (see 3.2.3). Cut-off values for sheep are: GRA6 II = 0.452; GRA6 I/III = 0.377; GRA7 I = 0.038; GRA7 III = 0.076; Am6 = 0.569; Am7 = 0.022; Af6 = 0.651.

Seven different serotype profiles were observed among thirty-five serum samples from chickens. Nearly one third of serum samples did not recognize any peptide (31.4%). Serotype II was found in nine (25.7%) chickens. Eight chickens (22.9%) only recognized the atypical peptides (ND). Two different mixed profiles or cross-reaction (CR) were found in three chickens (8.6%): II/I/III in two animals and II/III in one (Figure 31). These mixed profiles represents serum samples that have reacted with more than one peptide or a possible mixed infection.

Chickens (n = 35)

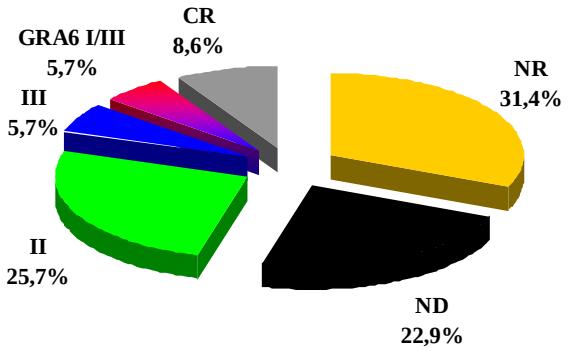


Figure 31: Serotype profiles found for thirty-five serum samples from chickens.

More than half serum samples from pigs (58.6%) did not recognize any peptide. Serotype II was found in six pigs (20.7%). Two different mixed profiles (CR) were found: II/I/III in five pigs and II/III in one animal (Figure 32).

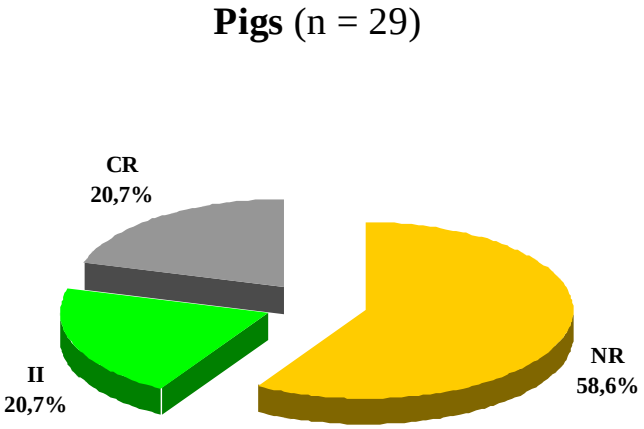


Figure 32: Serotype profiles found for twenty-nine serum samples from pigs.

Serotypes I, II and III were found in sheep. Serotype III was the most prevalent, being found in ten out of fifty sera (20%). Three sheep (6%) only recognized the atypical peptides (ND). Three mixed profiles (CR) were found in ten sheep (20%): I/III in two animals, II/I/III in five and II/III in three. Almost half serum samples from sheep (44%) did not recognize any peptide (Figure 33).

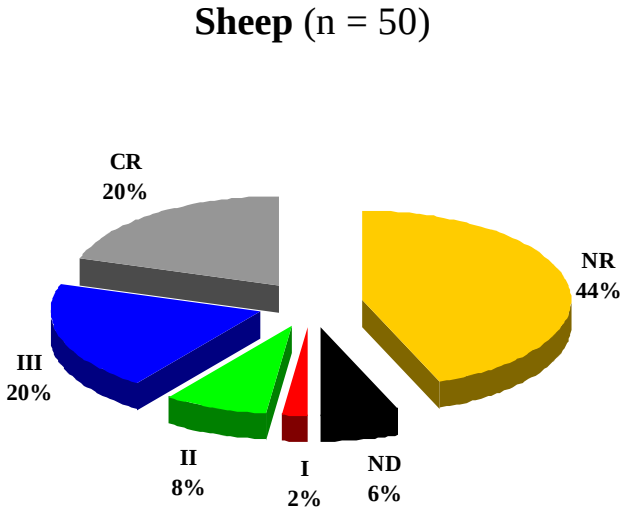


Figure 33: Serotype profiles found for fifty serum samples from sheep.

Between chickens and sheep a significant statistical difference was found for serotypes II and III ($p = 0.008$) and for serotypes III and ND ($p = 0.007$). For chickens, serotype II and ND were more frequent, while serotype III was more frequent in sheep.

3.2.5 Validation of archetypal strains specific peptides for serotyping of human sera

Serum samples from twenty-four cases of *Toxoplasma* infection with archetypal strains (I, II and III) from France and Portugal were studied with the peptides GRA6II, GRA6I/III, GRA7I and GRA7III. For each case of congenital infection, serum samples from the mother and the child were analyzed, except for infections associated with strains LGE-2004-ALB and LGE-2002-FAY . For a same case of congenital infection, not all serum samples were successfully serotyped. Serotyping results were in agreement with MS genotyping results for 21 out of 24 cases associated with strains belonging to the three main lineages (Appendix 5 – Table 5). However, for the infections caused by the type II strains LGE-01-1 and LGE-2005-FRA, serum samples from mothers cross-reacted with peptides GRA7I and with GRA7I and GRA7III respectively, suggesting a possible mixed infection. Serum sample from the child infected with the strain LGE-97-3 (type III) cross-reacted with

the peptide GRA7I. For one case of congenital infection due to a type II strain (LGE-01-4), serum samples from mother and newborn reacted with peptides specific for type I, II and III strains, indicating a possible mixed infection. Serum samples from the infection A and from the infection caused by the strain LGE-01-2 presented OD index under the cut-off established for the four peptides.

Peptide GRA6II reacted with twenty-four out of thirty-five serum samples from patients infected with strains type II and it was not recognized by serum samples from patients infected with strains type I and III (Figure 34). Peptide GRA6I/III reacted with all serum samples from patients infected with type I and III strains, but cross-reacted with two serum samples infected with one strain type II (Figure 35). Peptide GRA7I was not recognized by any serum samples from patients infected with type I strains (Figure 36). Peptide GRA7III reacted with three out of six serum samples infected with strains type III and cross-reacted with a serum infected with a strain type II (Figure 37).

Reactivity against these peptides of serum samples from sixteen patients infected with non-archetypal strains from Suriname and French Guiana was also tested (Appendix 5 – Table 5). Non-archetypal strains GUY-2002-KOE and GUY-2002-MAT share for the GRA6 C-terminal region the same polymorphisms as GRA6 type I and III strains. As a consequence, serum samples from these patients reacted with the peptide GRA6 I/III. The non-archetypal strain GUY-2004-AKO shares for the GRA6 C-terminal region the same polymorphisms as GRA6 type I and III strains except for position 224 (H instead of a R). Serum samples from this patient reacted with the peptide GRA6 I/III. Twelve patients from an outbreak of toxoplasmosis in Suriname were infected with the same strain (GUY-2004-TER) (Demar *et al*., 2007). This strain and the strain GUY-2003-BAS shares at the GRA6 C-terminal region 2 polymorphic residues with type II strains (G at position 223 and S at position 224) and 2 polymorphic residues with type I and III strains (V at position 227 and Y at position 230). Different responses were obtained for the patients infected with these two strains: exclusive reaction with GRA6 II in two patients, exclusive reaction with GRA6 I/III in two patients, reaction with both peptides in three patients, reaction with GRA6 II, GRA6 I/III and GRA7 III in one patient, reaction with the four peptides in one patient or no recognition of the peptides in four patients. These five non-archetypal strains shares for the GRA7 C-terminal region the same polymorphisms as GRA7 type I strains. Based on this, it would be expected

that serum samples from patients infected with these strains reacted with this peptide. However, only one patient infected with the strain GUY-2004-TER reacted with peptide GRA7I. Although peptide GRA7III differs by three amino acids from GRA7 C-terminal region of these strains, serum samples from three patients (two of them infected with the strain GUY-2004-TER and one with the strain GUY-2002-KOE) reacted with this peptide. These results demonstrate that peptides specific for the archetypal strains (I, II, III) cross-react with serum samples from patients infected with non-archetypal strains (Figures 34 to 37).

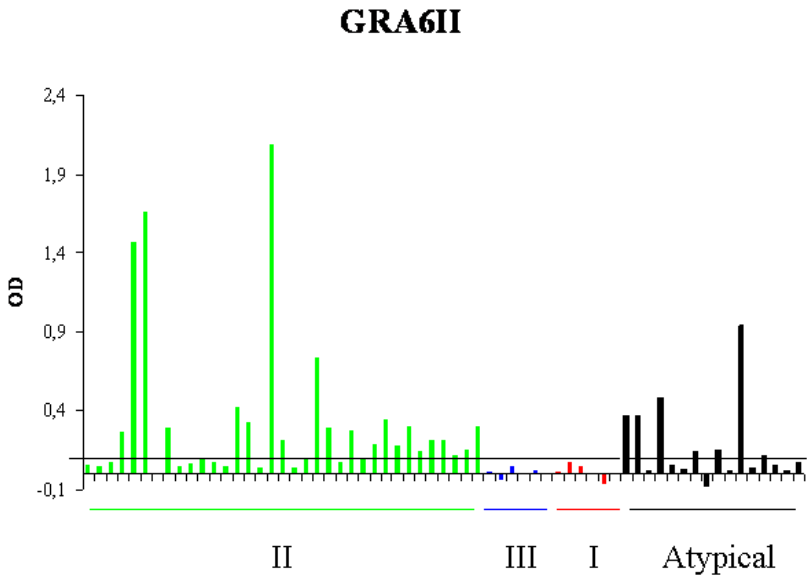


Figure 34: Pattern of reactivity of serum samples from patients infected with type I, II, III and atypical strains against peptide GRA6II. Cut-off for Europe (archetypal strains) is 0.088 and for South America (atypical strains) is 0.089 (horizontal line).

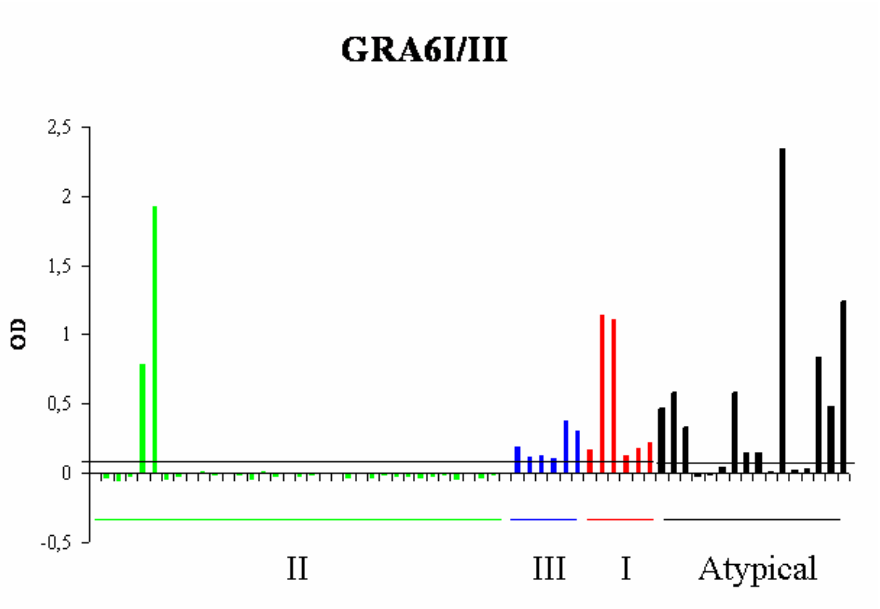


Figure 35: Pattern of reactivity of serum samples from patients infected with type I, II, III and atypical strains against peptide GRA6I/III. Cut-off for Europe (archetypal strains) is 0.076 and for South America (atypical strains) is 0.067 (horizontal line).

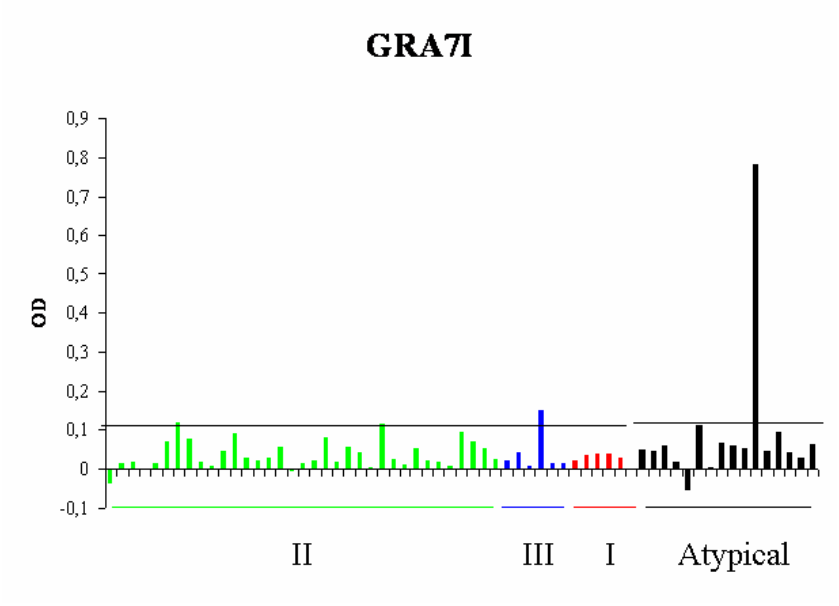


Figure 36: Pattern of reactivity of serum samples from patients infected with type I, II, III and atypical strains against peptide GRA7I. Cut-off for Europe (archetypal strains) is 0.114 and for South America (atypical strains) is 0.124 (horizontal line).

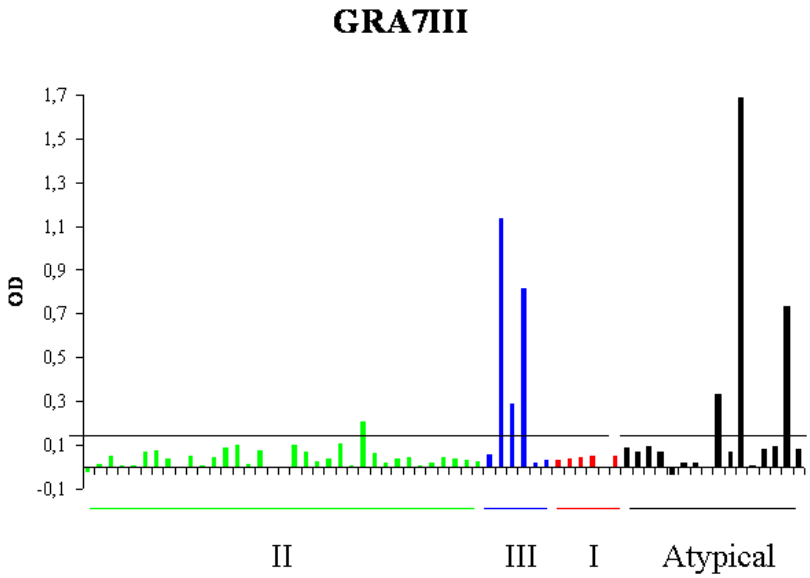


Figure 37: Pattern of reactivity of serum samples from patients infected with type I, II, III and atypical strains against peptide GRA7III. Cut-off for Europe (archetypal strains) is 0.126 and for South America (atypical strains) is 0.120 (horizontal line).

These four peptides showed a high specificity for serotyping strains belonging to the clonal lineages I, II and III. Peptide GRA6II had a specificity of 100%; peptide GRA6I/III a specificity of 94.3%; peptide GRA7I a specificity of 92.7%; and peptide GRA7III of 97.6%. Except peptide GRA6I/III, which had a sensitivity of 100% for identification of type I or III infections, the remaining peptides showed low sensitivity for serotyping infections. Peptides GRA6II and GRA7III had a sensitivity of 68.6% and 50% respectively. Peptide GRA7I did not recognize any type I infection (0% sensitivity). However, cross-reactivity observed between these peptides and serum samples from atypical infections diminish the specificity of GRA6II (75%), GRA6I/III (79.6%) and GRA7III (93%).

3.2.6 Validation of non-archetypal strains specific peptides for serotyping of human sera

Serum samples from patients infected with archetypal and non-archetypal strains were tested with three peptides (Am6, Am7 and Af6) specific for atypical strains (Appendix 5 – Table 6).

The non-archetypal strains GUY-2002-MAT and GUY-2002-KOE have seven residues of difference with peptide Am6 and only one with peptide Af6. Strain GUY-2004-AKO has six residues of differences with peptide Am6 and two with peptide Af6. Strains GUY-2003-BAS and GUY-2004-TER have five residues of difference with peptide Am6 and three with peptide Af6. Both peptides were recognized by serum samples from patients infected with these atypical strains (Figure 38 and 39). Eight and 11 out of 16 serum samples from these patients recognized the peptides Am6 and Af6 respectively. These five atypical strains share with peptide Am7 the same polymorphisms. Only 3 (18.8%) serum samples from 16 patients infected with atypical strains recognized the peptide Am7 (Figure 40).

These peptides were also tested against 10 serum samples from patients infected with archetypal strains (I, II and III). Peptide Am6 differs by 9 amino acids from Type I strains, by 4 aa from type II and by 8 aa from type III strains. This peptide was recognized by 8 out of 10 patients infected with the archetypal strains (Figure 38). Peptide Af6 differs by 4 amino acids from type I strains, by 12 aa from type II and by 3 aa from type III strains. This peptide was recognized by 7 out of 10 patients infected with these archetypal strains (Figure 39). Peptide Am7 did not reacted with any sera from patients infected with type I, II and III strains (Figure 40).

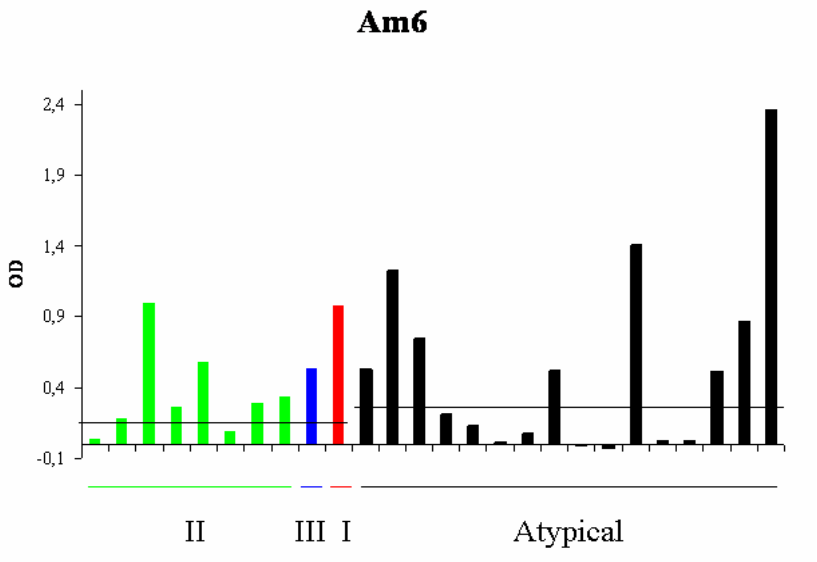


Figure 38: Pattern of reactivity of serum samples from patients infected with strains type I, II, III and atypical against peptide Am6. Cut-off for Europe (archetypal strains) is 0.180 and for South America (atypical strains) is 0.252 (horizontal line).

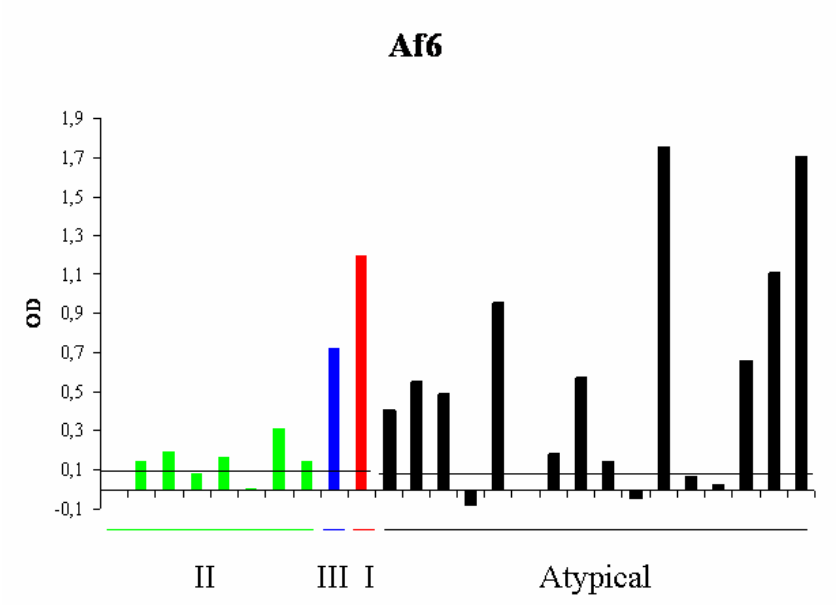


Figure 39: Pattern of reactivity of serum samples from patients infected with strains type I, II, III and atypical against peptide Af6. Cut-off for Europe (archetypal strains) is 0.096 and for South America (atypical strains) is 0.079 (horizontal line).

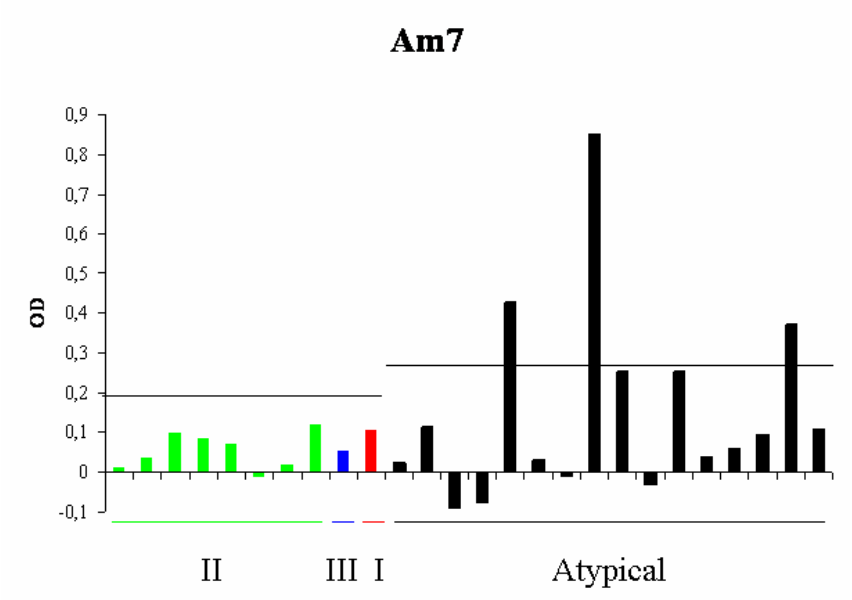


Figure 40: Pattern of reactivity of serum samples from patients infected with strains type I, II, III and atypical against peptide Am7. Cut-off for Europe (archetypal strains) is 0.188 and for South America (atypical strains) is 0.270 (horizontal line).

Am6 and Af6 are very reactive peptides, reacting with serum samples from infections associated with archetypal and atypical strains. Both peptides had a low specificity. Peptide Am6 had a specificity of 38.5% and peptide Af6 had a specificity of 30.8%. Peptide Am7 had a high specificity (100%), but a sensitivity of only 18.8%.

3.2.7 Geographical distribution of *Toxoplasma gondii* serotypes in humans

Serum samples from humans seropositive for *T. gondii* from three geographical distinct regions were serotyped based on the reactivity profile against GRA6 and GRA7 archetypal lineages specific peptides (GRA6II, GRA6I/III, GRA7I and GRA7III).

Serotype II profile was significantly more frequent ($p<0.05$) in serum samples from European infections with unknown genotype, being found in 48.4% of the studied patients (69.5% if we consider only sera for which serotyping was successful). Interestingly, it was noted that in Portugal, type III profile and mixed reaction with the different peptides were more common than in France, but this difference was not statistically significant ($p>0.05$). Type III profile was found in 7.9% of the Portuguese patients and in only 1.1% of the patients from France. Mixed profile was found in 15.1% of the Portuguese patients (I/III profile was found in 0.8%, II/I/III profile in 5.6%, II/I profile in 4%, and II/III profile in 4.8% of the patients) and in 9.6% of the French patients (I/III and II/III profile in 2.1% of the patients each and II/I/III profile in 5.3%) (Table 14 and Figure 41).

Table 14: Serotype distribution in Europe

Geographic region (n)	I (n)	II (n)	III (n)	GRA6I/III (n)	Cross-reaction or mixed profile				NR* (n)
					I/III° (n)	II/I/III# (n)	II/I∴ (n)	II/IIIϕ (n)	
France (94)	2.1% (2)	40.0% (38)	1,1% (1)	0	2.1% (2)	5.3% (5)	0	2.1% (2)	46.8% (44)
Portugal (126)	0	54% (68)	7.9% (10)	4.8% (6)	0.8% (1)	5.6% (7)	4% (5)	4.8% (6)	18.3% (23)
Denmark (1)	0	1	0	0	0	0	0	0	0
Europe (221)	0.9% (2)	48.4% (107)	5% (11)	2.7% (6)	1.4% (3)	5.4% (12)	2.3% (5)	3.6% (8)	31.3% (67)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.088; GRA6 I/III = 0.076; GRA7 I = 0.114; GRA7 III = 0.126).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

∴II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

ϕII/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

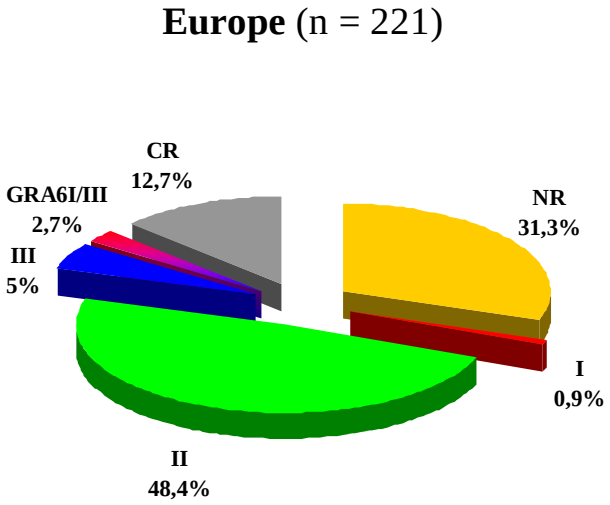


Figure 41: Serotype profiles found for 221 serum samples from Europe.

Serotype I and GRA6I/III were significantly more frequent in serum samples from Africa and Latin America ($p<0.05$) (Tables 15 and 16; Figures 42 and 43). In those regions, GRA6I/III profile was found in 19.8% and 30.6% respectively, but in only 2.7% of the patients in Europe. Serotype I profile was found in 8.1% and 10.2% for Africa and Latin America respectively, but only in 0.9% of the patients in Europe.

Some serum samples reacted with more than one peptide (CR) suggesting a possible mixed infection. This cross-reactivity was observed in serum samples from the three continents.

The number of serum samples that did not recognize these peptides (ND), varied between 22% and 34% in the 3 continents. In Africa and Latin America, respectively 33.7% and 22.3% of the cases were not serotyped. In Europe 31.3% of the cases were not serotyped. No relation was established between this lack of response, antibody titer and time of infection.

Table 15: Serotype distribution in Africa

Geographic region (n)	I (n)	II (n)	III (n)	GRA6I/III (n)	Cross-reaction or mixed profiles				NR* (n)
					I/III° (n)	II/I/III# (n)	II/IIIϕ (n)	II/I∴ (n)	
Ivory Coast (38)	7.9% (3)	5.3% (2)	10.5% (4)	15.8% (6)	2.6% (1)	15.8% (6)	0	2.6% (1)	39.5% (15)
Congo (17)	0	17.6% (3)	0	23.5% (4)	0	17.6% (3)	0	0	41.2% (7)
Angola (6)	16.7% (1)	16.7% (1)	0	16.7% (1)	0	16.7% (1)	0	0	33.3% (2)
Gabon (5)	20% (1)	0	0	60% (3)	0	0	0	0	20% (1)
African origin (20)	10% (2)	10% (2)	15% (3)	15% (3)	15% (3)	10% (2)	5% (1)	0	20% (4)
Total Africa (86)	8.1% (7)	9.3% (8)	8.1% (7)	19.8% (17)	4.7% (4)	14% (12)	1.2% (1)	1.2% (1)	33.7% (29)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.111; GRA6 I/III = 0.079; GRA7 I = 0.098; GRA7 III = 0.173).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

ϕII/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

∴II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

Africa (n = 86)

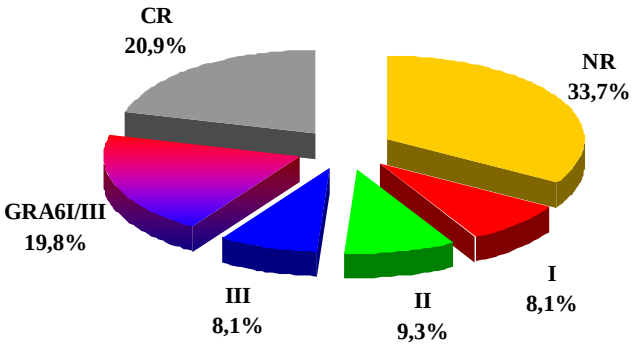


Figure 42: Serotype profiles found for 86 serum samples from Africa.

Table 16: Serotype distribution in Latin America

Geographic region (n)	I (n)	II (n)	III (n)	GRA6I/III (n)	Cross-reaction or mixed profile				NR* (n)
					I/III° (n)	II/I/III# (n)	II/I∴ (n)	II/IIIϕ (n)	
French Guiana (31)	6.5% (2)	6.5% (2)	9.7% (3)	25.8% (8)	0	22.6% (7)	6.5% (2)	0	22.6% (7)
Mexico (124)	11.3% (14)	3.2% (4)	12.9% (16)	32.3% (40)	3.2% (4)	11.3% (14)	0	3.2% (4)	22.6% (28)
Colombia (2)	0	50% (1)	0	0	0	50% (1)	0	0	0
Latin America (157)	10.2% (16)	4.5% (7)	12.1% (19)	30.6% (48)	2.5% (4)	14% (22)	1.3% (2)	2.5% (4)	22.3% (35)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.089; GRA6 I/III = 0.067; GRA7 I = 0.124; GRA7 III = 0.120).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

∴II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

ϕII/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

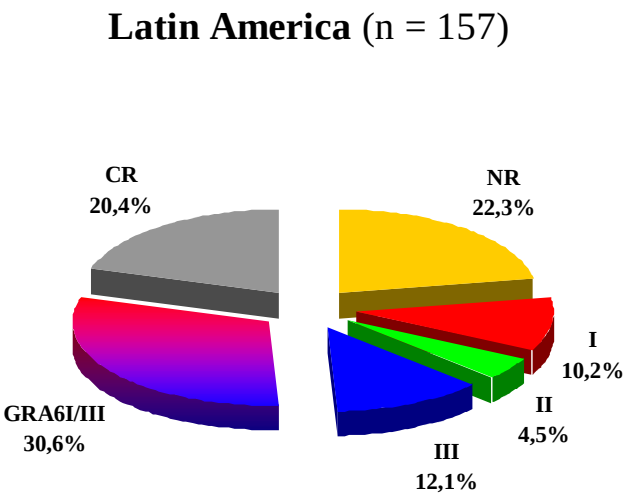


Figure 43: Serotype profiles found for 157 serum samples from Latin America.

3.2.8 Clinical aspects and serotype association

Serum samples from humans seropositive for *T. gondii* with asymptomatic infections and clinical manifestations were serotyped based on the reactivity profile against GRA6 and GRA7 archetypal lineages specific peptides (GRA6II, GRA6I/III, GRA7I and GRA7III).

Serotype II profile was the most frequently found in serum samples from symptomatic and asymptomatic patients from Europe (Table 17). The two cases of cerebral toxoplasmosis and three out of four patients with ocular infection were associated to serotype II strains. This same serotype was found in 63.3% of the congenital infections. Congenital infections associated with serotype III or with a mixed profile (CR) were only found among Portuguese patients. Among French patients, serotype III and mixed profiles (CR) were only found for asymptomatic patients (immunocompromised and immunocompetent). There was no marked difference in the distribution of serotypes according to the different categories of patients (congenital versus asymptomatic, immunocompromised asymptomatic versus immunocompetent asymptomatic) in Europe (p>0.05).

Table 17: *T. gondii* serotypes and association with clinical aspects in Europe

Pathology (n)	**I (n)**	**II (n)**	**III (n)**	**GRA6I/III (n)**	**Cross-reaction or mixed profile**				**NR* (n)**
					I/III° (n)	**II/I/III# (n)**	**II/I∴ (n)**	**II/IIIϕ (n)**	
Congenital (44)	0	63.6% (28)	6.8% (3)	2.3% (1)	0	2.3% (1)	2.3% (1)	2.3% (1)	20.5% (9)
Cerebral (2)	0	2	0	0	0	0	0	0	0
Pulmonary (1)	0	0	0	0	0	0	0	0	1
Ocular (4)	0	75% (3)	0	0	0	0	0	0	25% (1)
Immunocompromised asymptomatic (31)	0	48.4% (15)	6.5% (2)	0	3.2% (1)	12.9% (4)	3.2% (1)	3.2% (1)	22.6% (7)
Immunocompetent asymptomatic (130)	1.5% (2)	38.5% (50)	4.6% (6)	3.8% (5)	1.5% (2)	5.4% (7)	2.3% (3)	4.6% (6)	37.7% (49)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.088; GRA6 I/III = 0.076; GRA7 I = 0.114; GRA7 III = 0.126).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

∴II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

ϕII/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

The reduced number of samples from symptomatic patients from Africa and Latin America did not allow us to make a reliable statistical analysis of the serotype frequency for different pathologies.

Except for one case of congenital toxoplasmosis and one ocular infection, all serum samples from Africa belonged to asymptomatic patients. The profile GRA6I/III was the most frequent, being found in 37.5% of the immunocompromised patients and in 18.4% of the immunocompetent patients. Serotype II was only found in immunocompetent asymptomatic patients (9.2%) and for the only case of congenital infection from Africa (Table 18).

Table 18: *T. gondii* serotypes and association with clinical aspects in Africa

Pathology (n)	I (n)	II (n)	III (n)	GRA6I/III (n)	Cross-reaction or mixed profile				NR* (n)
					I/III° (n)	II/I/III# (n)	II/III$^{\phi}$ (n)	II/I$^{\therefore}$ (n)	
Congenital (1)	0	1	0	0	0	0	0	0	0
Ocular (1)	0	0	0	0	0	0	0	0	1
Immunocompromised asymptomatic (8)	12.5% (1)	0	12.5% (1)	37.5% (3)	12.5% (1)	12.5% (1)	12.5% (1)	0	0
Immunocompetent asymptomatic (76)	7.9% (6)	9.2% (7)	7.9% (6)	18.4% (14)	3.9% (3)	14.5% (11)	0	1.3% (1)	36.8% (28)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.111; GRA6 I/III = 0.079; GRA7 I = 0.098; GRA7 III = 0.173).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

$^{\phi}$II/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

$^{\therefore}$II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

GRA6I/III profile was the most frequently found for asymptomatic (31.1%) patients from Latin America (Table 19). Serotype I was found for the only case of congenital toxoplasmosis and in 9.9% of the asymptomatic patients. Serotype II was found in one case of severe multivisceral infection, one case of ocular infection and in five (3.3%) asymptomatic

patients. Mixed II/I/III profile was found in one case of acute infection in an immunocompetent patient and in 13.9% of the asymptomatic patients.

Table 19: *T. gondii* serotypes and association with clinical aspects in Latin America

Pathology (n)	I (n)	II (n)	III (n)	GRA6I/III (n)	Cross-reaction or mixed profile				NR* (n)
					I/III° (n)	II/I/III# (n)	II/I∴ (n)	II/IIIϕ (n)	
Congenital (1)	1	0	0	0	0	0	0	0	0
Ocular (1)	0	1	0	0	0	0	0	0	0
Acute infection (1)	0	0	0	0	0	1	0	0	0
Multivisceral (3)	0	1	0	1	0	0	0	0	1
Immunocompetent asymptomatic (151)	9.9% (15)	3.3% (5)	12.6% (19)	31.1% (47)	2.6% (4)	13.9% (21)	1.3% (2)	2.6% (4)	22.5% (34)

*NR (not reactive), serum samples with OD below cut-off values (GRA6 II = 0.089; GRA6 I/III = 0.067; GRA7 I = 0.124; GRA7 III = 0.120).

°I/III, serum samples that reacted with GRA7I and GRA7III.

#II/I/III, serum samples that reacted with GRA6II, GRA6I/III, GRA7I and GRA7III.

∴II/I, serum samples that reacted with GRA6II, GRA6I/III and GRA7I or with GRA6II and GRA7I.

ϕII/III, serum samples that reacted with GRA6II, GRA6I/III and GRA7III or with GRA6II and GRA7III.

3.2.9 Reactivity against atypical peptides

In order to test the reactivity of the peptides specific for non-archetypal strains with serum samples, sixty-six sera from France and Portugal and the thirty eight sera collected from asymptomatic patients from Ivory Coast were studied. Reactivity against these peptides was higher among serum samples from Ivory Coast than in European countries ($p<0.05$) (Table 20). Only seven out of twenty-four serum samples from Portugal (29.2%) and ten out of forty-two sera from France (23.8%) reacted with at least one of the atypical peptides. Serum samples from Europe positive for these peptides were also reactive against one or more archetypal peptides (Appendix 5 - Table 7). Twenty-six serum samples from Ivory Coast (68.4%) reacted with one, two or the three non-archetypal peptides. Fifteen out of 26 sera (57.7%) from Ivory Coast that reacted with non-archetypal peptides also reacted with the peptides specific for the archetypal strains. The remaining eleven samples reacted exclusively with the atypical peptides (Appendix 5 - Table 7). Only serum samples from Ivory Coast reacted with the peptide Am7.

Serum samples from Europe belonged to patients with congenital infections or to asymptomatic patients (immunocompromised and immunocompetent). Reaction against atypical peptides was more prevalent in immunocompromised patients (72.7% of eleven) than in immunocompetent (17.9% of thirty-nine, $p>0.05$) and congenital infections (14.3% of fourteen, $p<0.05$) (Appendix 5- Table 7).

Table 20: Reactivity of serum samples from different geographic regions against atypical peptides.

Geographic origin	**Studied sera**	**Positive sera**		
		Am6	**Am7**	**Af6**
France	42	10	0	9
Portugal	24	2	0	7
Ivory Coast	38	23	12	11

3.2.10 Serotype characterization of *Toxoplasma* infection in patients from Uruguay

Two hundred and eight patients positive for *T. gondii* were serotyped. Twelve serum samples from individuals seronegative for *T. gondii* were used to establish the cut-off values for the seven studied peptides (GRA6II, GRA6I/III, GRA7I, GRA7III, Am6, Am7 and Af6). Patients were considered positive for these peptides when OD index was equal or higher than established cut-off (0.061, 0.014, 0.496, 0.277, 0.119, 0.769 and 0.101 respectively). Seven different profiles were found. Serotypes II and III were found in 28.4% (59 patients) and 3.8% (8 patients) respectively. Fourty-eight patients (23.1%) had a GRA6I/III profile. Two different mixed profiles (CR) were found for 53 patients (25.5%). Mixed profile II/I/III was found in 36 patients (17.3%) and mixed profile II/III was found in seventeen (8.2%). Ten patients (4.8%) reacted exclusively with the atypical peptides (ND). Serum samples from thirty patients (14.4%) did not recognized any peptide (NR).

In order to evaluate if the serotype distribution varies according to geography inside the country, sera from different regions, more or less related to trade exchange with Europe, were analyzed (Figure 44). North region includes serum samples collected from Artigas, Riviera, Rio Negro and Tacuarembo. Central region includes serum samples collected from Cerro Largo, Durazno, Flores and Trienta y tres. South region includes serum samples collected from Colonia, Canelones, Lavalleja, Maldonado, Rocha, Florida, San José and Montevideo.

Figure 44: Map of Uruguay. Regions from which serum samples were collected are marked.

Results obtained for each one of these regions showed differences mainly in the distribution of serotype II, III, profile GRA6I/III and mixed reactions. Profile GRA6I/III and serotype II were more frequently found in the South, while mixed infections (CR) were more frequent in the North. Differences for serotype II were statistically significative (p=0.011) (Table 21). Similar results were obtained when serum samples from Montevido were excluded from South region group. (Figures 45 to 48).

Table 21: Frequencies of serotype according to geography.

Serotype	**North** n= 41	**South** n=132	**Center** n=35	**Significance** p
II	6 (14.6%)	37 (28.0%)	16 (45.7%)	**0.011**
III	3 (7.3%)	5 (3.8%)	0	0.129
GRA6I/III	8 (19.5%)	37 (28.0%)	3 (8.6%)	0.208
Cross-reaction	16 (39.0%)	27 (20.5%)	10 (28.6%)	0.182
Non reactive	8 (19.5%)	20 (15.1%)	2 (5.7%)	0.351
Not determined	0	6 (4.5%)	4 (11.4%)	0.393

North Uruguay (n=41)

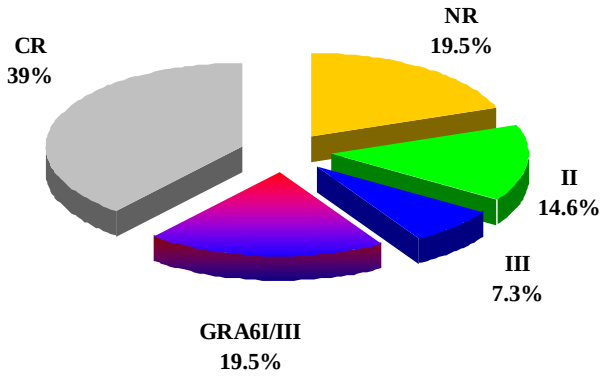


Figure 45: Serotype profiles found for forty-one serum samples from North Uruguay.

Uruguay Central Region (n=35)

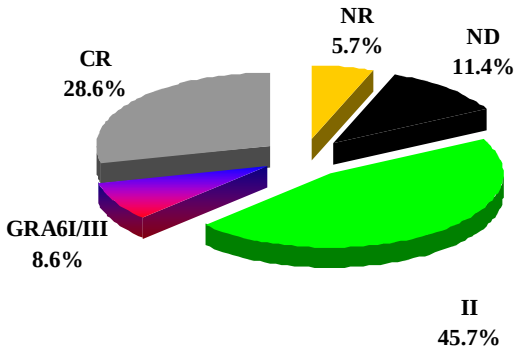


Figure 46: Serotype profiles found for thirty-five serum samples from Uruguay central region.

South Uruguay (n=132)

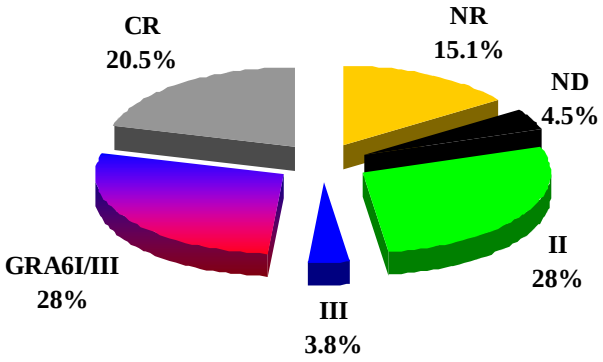


Figure 47: Serotype profiles found for one hundred and thirty-two serum samples from South Uruguay.

South Uruguay (excluding Montevideo)
(n=46)

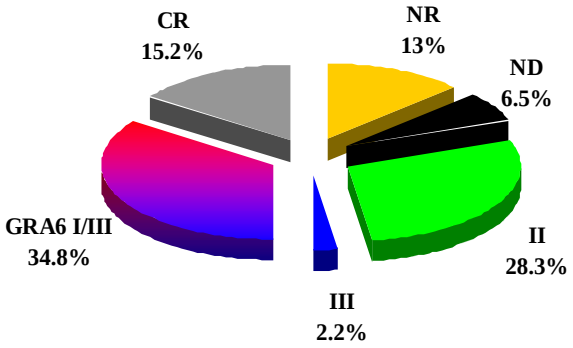


Figure 48: Serotype profiles found for forty-six serum samples from South Uruguay, excluding Montevideo sera.

Results obtained with serum samples from South region (excluding Montevideo) and serum samples from Montevideo were similar except for profile GRA6I/III and mixed infections. GRA6I/III profile was more frequent in the South (excluding Montevideo), while mixed infections were more frequent in Montevideo. However, this difference was not statistically significant (Figures 48 and 49).

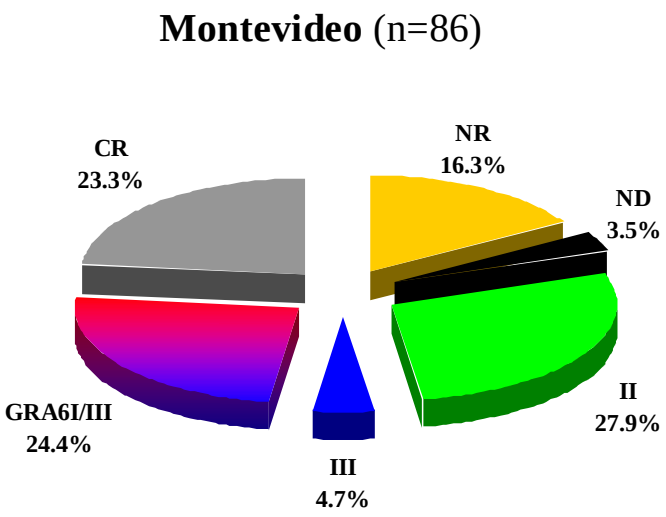


Figure 49: Serotype profiles found for eighty-six serum samples from Montevideo.

Regarding Montevideo region, no significative difference was found between the urban and suburban region except for mixed infections (CR) and ND serotypes. Mixed infections were statistically more frequent in the suburban area, while ND serotypes were more frequent in the urban region ($p < 0.05$) (Figures 50 and 51).

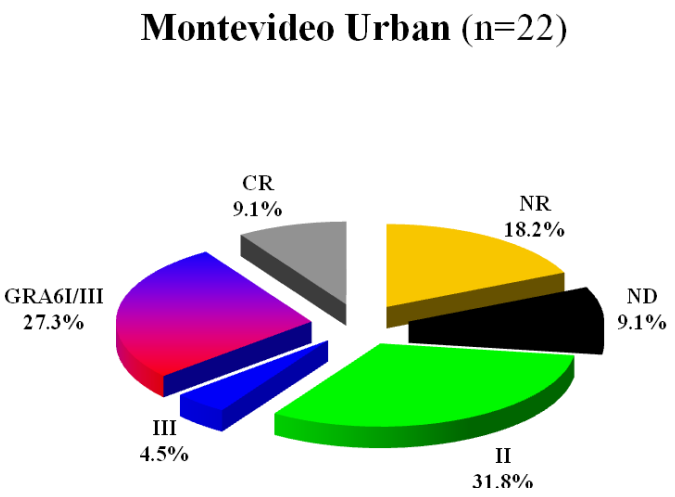


Figure 50: Serotype profiles found for twenty-two serum samples from Montevideo urban.

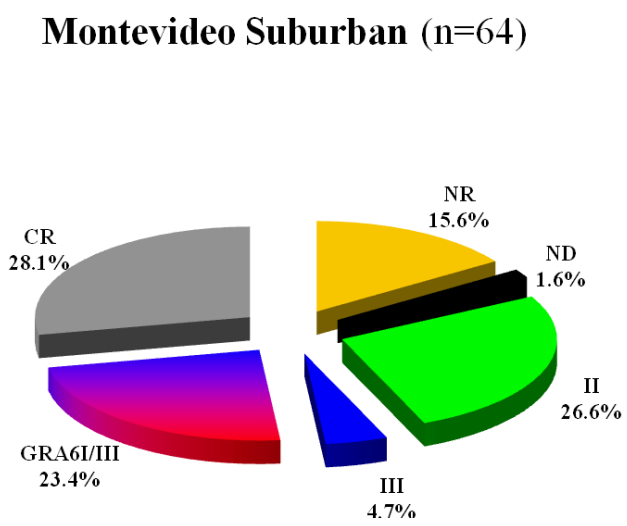


Figure 51: Serotype profiles found for sixty-four serum samples from Montevideo suburban.

Part IV

Discussion

Toxoplasma gondii is a parasite that occurs in most parts of the world, affecting both animals and humans. Toxoplasmosis is considered the third cause of death associated with food-borne infections in Europe (EFSA, 2007) and USA (Mead *et al*., 1999). Outbreaks of toxoplasmosis have been described associated to the consumption of raw meat and contaminated water. These outbreaks were usually associated with cases of severe acute infection in immunocompetent patients. The genetic characterization of *Toxoplasma* strains associated with these outbreaks from other continents than Europe and North America revealed that genetic diversity of *T. gondii* is higher than initially proposed. Genetic characterization of *T. gondii* strains is an essential tool for the knowledge of the parasite population and to understand how the strain genotype may influence the disease burden in the host. Genotyping methods require the previous isolation of the parasite or parasite DNA. This limits the studies, especially in humans, to *Toxoplasma* infections with clinical manifestations. In order to understand the real genetic diversity of *T. gondii* and in which way it is associated with disease manifestations, studies should be conducted in symptomatic and asymptomatic chronic infections.

The main objective of this work was to develop a genotyping method based on specific antigen-antibody recognition, which does not require previous isolation of the parasite. This will eliminate the biases associated with genotyping based on parasite isolation. The only biological material required for serotyping is serum samples, which can be easily obtained from both symptomatic and asymptomatic patients. Besides, infections with different strains are usually not detected by the murine bioassay since only one strain is isolated. The different antibodies produced against different strains will recognize strain-specific antigens allowing to detect mixed infections. In this method strain-specific synthetic peptides are specifically recognized by the antibodies. The peptides described so far have a limited utility. Those peptides only distinguished type II strains from non-type II. As consequence, it is not possible to differentiate the clonal types I and III, as well as all the non-archetypal genotypes.

Kong and colleagues (2003) selected and tested a high number of *T. gondii* antigens, which included GRA, SAG, ROP, NTP, BSR and SRS proteins. From all the tested peptides, only a few number derived from GRA6 and GRA7 proteins were recognized by human serum samples. Antigens selected for serotyping must be immunogenic and polymorphic. The antigens must induce a specific antibody response and must have strain-specific

polymorphisms. Otherwise, selected peptides will not be recognized in a strain-specific manner. A search in the literature was performed in order to find other immunogenic and polymorphic antigens usable in serotyping. Most of the antigens that gathered these two properties have been already described with no promising results (Kong *et al*., 2003). GRA8 antigen is an immunogenic protein (Beghetto *et al*., 2003) and was already described for genotyping of *T. gondii* (Lin *et al*., 2005). Sequencing of a *T. gondii* strain from Korea revealed that GRA8 possess two nucleotide and two amino acids substitutions when compared with RH strain (Lin *et al*., 2005). Based on these previous findings, GRA8 was also selected for sequencing.

Peptides derived from GRA5 were also tested for serotyping (Morriset *et al*., 2008; Peyron *et al*., 2006). Peptides were derived from the hydrophilic N-terminal region (the first 75 aa), where all but one polymorphic residues are found for the types I, II and III. The GRA5 peptides clearly differentiate type II from types I and III. This antigen was not included in this study, since GRA5 peptides specific for type I and type III strains were less reactive than GRA6 derived peptides (Peyron *et al*., 2006).

4.1 Sequencing analysis of *Toxoplasma gondii* strains

The coding region for *GRA6* has already been described as highly polymorphic (Fazaeli *et al*., 2000). In Fazaeli *et al*. (2000), nine different alleles were identified by sequencing within 30 strains. Other *GRA6* alleles were found in type X and type A, two genotypes described in Californian sea otters (Conrad *et al*., 2005; Miller *et al*., 2004; Miller *et al*., 2008a; Sundar *et al*., 2008). The allele X for *GRA6* was also found in a *Toxoplasma* isolate from a free-living jaguar from French Guiana (Demar *et al*., 2008). Zakimi and colleagues (2006b) found nine new sequences among 29 isolates from Japanese pigs. Our sequence analysis is in agreement with these previous studies and confirms the high polymorphism of the *GRA6* gene. Nineteen different *GRA6* haplotypes were found within 49 strains already typed as non-archetypal by a multilocus analysis with 5 microsatellites (*TUB2*, *TgM-A*, *W35*, *B17*, and *B18*). Six *GRA6* haplotypes (1, 2, 3, 4, 5 and 11) found in our strains have also been described by Fazaeli and colleagues (2000). The nine new sequences found in pigs from Japan (Zakimi *et al*., 2006b) were not found in our strains.

Few studies regarding *GRA7*, *GRA8* and genotyping have been conducted. Except for the three archetypal lineages (I, II, III), none of the haplotypes described for *GRA7* had been already published. However, *GRA7* PCR-RFLP is routinely used for genotyping by the French National Reference Center for Toxoplasmosis and Biological Resource Center for Toxoplasma (BRC) (http://www.toxobrc.com/). This locus does not present the same degree of polymorphism than *GRA6*. *GRA8* locus, although only sequenced for 19 strains, clearly show very few polymorphisms. *GRA8* was successfully amplified and sequenced for the strains belonging to clonal types I and III, and non-archetypal strains. However, it was not possible to amplify any type II strain. Analysis of *GRA8* sequence from the strain Me49 (type II) published on ToxoDB (http://toxodb.org/toxo/) showed that this strain has a *GRA8* nucleotide sequence with polymorphisms specific for this strain and the existence of three polymorphic positions in the region selected for primer reverse. These polymorphisms may justify the amplification problems of strains type II with the primers described by Lin *et al*. (2005). Although immunogenic, this locus is not sufficiently polymorphic to be used in serotyping.

Phylogenetic analysis of *GRA6* and *GRA7* loci showed that most strains are clustered by geographic region (Africa, French Guiana and Brazil). Strains from Africa are close to type I strains, while the majority of strains from Brazil are close to type III strains. Strains from French Guiana form a separated branch with other atypical strains, yet closer to type I strains. The fact that most of the studied strains had South American origin (37 strains), and only a few numbers were from other geographical regions (six from Africa, five from Europe and three from USA) may have introduced bias in the phylogenetic analysis. For the two loci, exclusive haplotypes were found for South American strains, being the rest of them shared by the different geographical regions. These results are in agreement with recent *Toxoplasma* population studies. Lehmann and colleagues (2006) defined four populations for *Toxoplasma*. Two populations were exclusive from South America; one was shared by Europe, North America, Asia and Africa and a fourth population had worldwide distribution. More recently, four clonal lineages were defined for Brazilian strains (Pena *et al*., 2008). It was not possible to identify these Brazilian clonal lineages among the strains included in this study, due to the lack of common strains and markers between the two studies. Our haplotypes were defined based on sequencing results on only two loci, while in the study conducted by Pena and colleagues (2008), the clonal lineages were defined based on a multi-locus RFLP study.

According with the study performed by Lecordier and colleagues (2000), only the N-terminal hydrophilic region of *GRA6* is recognised by *Toxoplasma* positive human sera. An ELISA based on the GRA6 C-terminal showed a sensitivity of 10% while a GRA6 N-terminal-based ELISA showed a sensitivity of 96%. The authors suggested that a major B-cell epitope(s) is carried by the N-terminal peptide portion of the *GRA6* antigen. The most important antigenic region of GRA7 seems to be located between amino acids 97 and 146 (Jacobs *et al*., 1999). According to these authors, the major hydrophilic domain in the C-terminal region does not contain an important epitope. However, the C-terminal region of these two proteins presents more polymorphisms than the N-terminal region. The results obtained by Kong and colleagues (2003) showed that the C-terminal region of GRA6 and GRA7 is probably also immunogenic, since the peptides selected from this region reacted extremely well with human sera. Despite the absence of an infallible method to predict antigenic peptides, there are several rules that can be followed to predict which regions of a protein are likely to be antigenic. Antigenic peptides should be located in solvent accessible regions and are usually located on the protein surface. Charged and hydrophilic amino acids are frequently presents in antigenic regions. Peptides should be selected from loops connecting secondary structure motifs avoiding peptides located in helical regions. Peptides should be selected from the N- and C-terminal regions, because these regions are usually solvent accessible and unstructured (MIF Bioinformatics – http://immunax.dfci.havard.edu/tools/antigenic.html). Kong and colleagues (2003) selected peptides based on the content of charged and hydrophilic amino acids, a predicted α-helix structure, the presence of proline residues and the presence of short regions bounded by 2 cysteine residues. In these work, the prediction of antigenic epitopes was based on the following parameters: hydrophobicity (Eisenberg *et al*., 1984), hydrophilicity (Hopp and Woods, 1981) and antigenicity (Welling *et al*., 1985) studies (ExPASy Proteomics server - www.expasy.org/tools/protscale.html). The proteins were also analysed using the Bioinformatics software (http://immunax.dfci.havard.edu/tools/antigenic.html) were the antigenic determinants were predicted according to the parameters defined above. Hopp and Woods (1981) defined a method to find the point of greatest hydrophilicity. The point of highest local average hydrophilicity is invariably located in, or immediately adjacent to an antigenic determinant. However, not all antigenic regions are hydrophilic and not all hydrophilic regions are antigenic (Hopp and Woods, 1981). Welling *et al*. (1985) developed a method for predicting antigenicity based on the percentage of each amino acid present in

known antigenic determinants compared with the percentage of the amino acids in the average composition of the protein. The regions selected as potential antigenic were crossed with amino acid polymorphic positions in order to define potential antigenic peptides. For both antigens (GRA6 and GRA7) the region C-terminal was selected as antigenic and was very polymorphic.

The peptides described by Kong and coleagues (2003) were selected from this region and comprised the last 4 polymorphic sites (223, 224, 227, and 230). If we consider only the peptide sequences chosen in this study, six different peptides can be described in the studied sample. Two peptides could differentiate type II (GS-E-F) from type I and III (ER-V-Y) as described by Kong and colleagues (2003). Three other peptides identified in non archetypal strains differed from peptide characterizing type I and III by a single aa at position 224 (EH-V-Y and ES-V-Y instead of ER-V-Y) or at position 227 (ER-E-Y instead of ER-V-Y). This single aa substitution may not be enough to distinguish *Toxoplasma* infection due to these strains and could explain that South American or African sera reacted as type I and III sera (Morisset *et al*., 2008; Peyron *et al*., 2006). The last peptide (GS-V-Y) is a mixture of aa sequences characterizing type II and types I and III that could explain cross-reactivity misleading to a conclusion of mixed infection (Kong *et al*., 2003; Peyron *et al*., 2006). On the contrary, these peptide polymorphisms may also partially explain, together with individual immunological reactivity, negative results of serotyping in South American countries where strain diversity is higher than in Europe.

At least 3 aa substitutions are generally needed to antibody-specific peptide differentiation. If two other close polymorphic positions of GRA6 antigen (218 and 219) were included, the resulting peptide will differentiate haplotype 1 (includes type I strains) (peptide [RP–ER–V–Y]) from haplotype 3 (includes type III strains) (peptide [RA–ER–V–Y]). However, this single aa substitution (proline replaced by alanine) may not be enough to distinguish *Toxoplasma* infection with these strains. If we extend the selected peptides to aa 198-230, three aa substitutions are found between haplotype 1 and haplotype 3. However the large number of identical aa can increase cross-reactions between these two peptides. Similarly, most non-archetypal strains found in South America or in Africa are characterized by peptides differing from type I or III by a maximum of three non consecutive aa with a large number of identical aa which may limit the use of these peptides for serotyping.

Regarding GRA7 sequences, the most polymorphic region is located between residues 170 and 182. Six different peptides were found for that region. Haplotype 1 and haplotype 2 (the two peptides differ by 3 aa), characteristic of type I and II strains respectively can be differentiated, as well as haplotype 5 and 14. Peptide [T–D–S–G–S] corresponding to haplotype 3 (includes type III strains) is also shared by haplotypes 4, 12, 11, 13, 7 and 8. It differs from peptide for haplotype 1 by three aa and for haplotype 2 by two aa. This peptide as well as peptide [A–E–T–S–N] and [A–D–T–S–N] may be important to differentiate strains from South America. Peptide [A–E–T–S–N] characteristic of haplotypes 6, 9 and 10 may be useful to differentiate strains from French Guiana. In this peptide region, African strains are mainly characterized by a type I peptide. This region was not selected by the antigenicity prediction studies neither by Kong and colleagues (2003). The peptides selected by Kong and colleagues (2003) were located in the C-terminal region of GRA7 (aa 225-236) and comprised 2 polymorphic aa (229, 231). We proposed to increase the size of this peptide, by including two other polymorphic aa (220, 222). In this region (220-236), only 3 different peptides can be selected that distinguish haplotypes 1, 2 and 3, characteristics of type I, II and III strains respectively. Atypical strains share the same polymorphism as type I and III strains. Peptides selected from this region may be good candidates to differentiate type I from type III (3 different aa) as well as type II from type III (4 different aa) strains.

4.2 Serotyping protocol

Serotyping studies conducted until now only distinguished type II from non-type II strains (Kong *et al.*, 2003; Morrisset *et al.*, 2008; Nowakoska *et al.*, 2006; Peyron *et al.*, 2006). In those studies, two different approaches were developed: synthetic peptides coupled to a carrier protein (Kong *et al.*, 2003) or recombinant peptides (Peyron *et al.*, 2006). The use of carrier proteins has been described in immunogenicity assays. Synthetic peptides coupled to carrier proteins are a useful tool for the generation of antibodies against specific peptides. Peptides due to its small size, fail to elicit a strong antibody response. They are able to bind to B-cells but are too small to contain motifs required to associate with MHC class II molecules and T-cells receptors. The carrier proteins coupled to the peptides provide the additional motifs and generate a strong antibody response. The most common carrier proteins are bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). The use of synthetic peptides as ELISA antigens can be problematic due to the small size of the peptides. Peptides of small

size will be difficult to attach to the plate and consequently the sensitivity of the assay will be lower. To cross this problem and to facilitate peptide attachment to the plate and consequently improve ELISA sensitivity, small peptides can be coupled to carrier proteins. In the present study, synthetic peptides were not coupled to a carrier protein and no cysteine residue was added to the coupling terminal. Peptides were coupled to the Immobilizer Amino plates (Nunc). These plates are manufactured using a photochemical method for covalent coupling of ligands to polymer materials. The AQ-Immobilizer reagent consists in the anthraquinone molecule linked to an electrophilic group through an ethylene glycol spacer. The electrophilic group reacts with nucleophiles such as free amines, thiols or hydroxyl-groups. These plates are specially adapted for small molecules, like peptides. The strong covalent binding of the anthraquinone to the microwell plate guaranties, that there will be no leaching of the bound molecule. The use of these plates replaces the role played by the carrier proteins. Besides these modifications, another different approach was proposed in our study and consisted on a trimer of the chosen peptide, i.e. a 3-times repeat in one single peptide of the chosen sequence (if smaller than 20 aa). This would increase the antigen size, with the objective of facilitating attachment to microplates and the antibody recognition. All studied peptides were synthesized as single sequence and as trimer as well. The first serotyping assays were made using each peptide sequence both as single and as trimer. These assays revealed that sequences with less than 20 aa had a higher sensitivity when synthesized as a trimer, while bigger sequences had a higher discriminative power, with better sensitivity and specificity when synthesized as a single sequence. Based on this, it was defined that sequences with less than 20 aa were synthesized as a trimer in one single peptide. The first serotyping assays were made with the objective of define the ELISA conditions. Peptide concentration, sera and conjugate dilutions, washing and dilution buffers and incubation times were the parameters tested. Cut-off values were defined for each species by studying serum samples that were IgG negative by MAT. For humans, cut-off values were established by geographic region. Cut-off values obtained for mice and domestic animals were for most peptides higher than for humans, which can be explained by the use of HRP-labelled conjugates in mice and domestic animals ELISA. HRP-labelled conjugates are less stable and give higher OD indexes, while alkaline-phosphatase conjugates (used for humans) are more stable and consequently the OD indexes obtained are lower. Except for mice and sheep, cut-off values for peptides GRA6II and GRA6I/III were lower than for the other peptides. Differences between cut-off values defined for each

continent were not relevant, except for peptide Af6, with a cut-off extremely high for Africa when compared with the values defined for Europe and Latin America.

Seven of the proposed peptides were synthesized and tested in order to evaluate their usefulness in serotyping assays. Peptides were selected from the C-terminal region of GRA6 and GRA7 antigen, and from the region between aa 170 and 182 of GRA7. For GRA6, this region comprises the sequence between aa positions 220 and 230, where four polymorphic aa are present at positions 223, 224, 227 and 230, for the peptides specific to the clonal lineages. Peptide GRA6 type II has polymorphisms characteristics of type II strains (GSEF). Peptide GRA6 type I/III has specific polymorphisms (ERVY) of type I and type III strains, since these strains share the same polymorphisms at the GRA6 C-terminal region. These peptides have already been tested by Kong and colleagues (2003), but modified with a cysteine residue in the N- or C-terminal region and coupled to a carrier protein. Two peptides specific for atypical strains (Am6 and Af6) were selected from the GRA6 C-terminal region between aa 198 and 230, as this region was polymorphic and possible immunogenic. Peptide Am6 has polymorphisms specific for strains belonging to haplotype 7. Peptide Af6 has polymorphisms specific for strains belonging to haplotype 11. Haplotype 11 was the most representative among African strains, being found in four out of six strains. Haplotype 7 was not exclusive of South American strains, since it was found for P80, a strain from USA. However, in comparison with the archetypal lineages and with the haplotype 11, this peptide has more polymorphisms than peptides derived from haplotypes exclusive from South America (6, 8, 9, 10, 16, 17). As consequence a better differentiation was expected. For GRA7, this region comprises the sequence between aa positions 220 and 236, where four polymorphic aa are present at positions 220, 222, 229 and 231. Peptide GRA7I has specific polymorphisms (LQEG) of type I strains. Peptide GRA7III has specific polymorphisms (PHER) of type III strains. At this region, peptides specific for type III strains selected by Kong and colleagues (2003) only comprised two polymorphic positions (229 and 231 or 220 and 222). One peptide specific for GRA7 type I strains which had two polymorphic positions at aa 220 and 222 was selected by Kong and colleagues (2003). This peptide is also shared by GRA7 type II strains. The other GRA7 type I specific peptide at C-terminal region selected by these authors comprised the polymorphic aa 229 and 231. These two peptides specific for GRA7 type I strains were only recognized by some of the serum samples studied by Kong and colleagues (2003). A third peptide was selected from GRA7, peptide Am7. This peptide has

polymorphisms specific of atypical strains from French Guiana with the haplotypes 6, 9 and 10.

4.3 Validation of serotyping protocol in mice and humans

In order to validate the serotyping protocol with the peptides GRA6II, GRA6 I/III, GRA7I and GRA7 III, serum samples from experimentally infected mice and human patients infected with different strains of *T. gondii* were tested against those peptides.

Peptide GRA6II was specifically recognized by mice infected with type II strains. It was expected that peptide GRA6I/III recognized serum samples from mice infected with strains type I and III. Although, specifically recognized by mice infected with strains type III, this peptide was not recognized by mice immunized with type I strain lysate. Similarly, serum samples from mice immunized with type I strains lysate did not reacted with the peptide GRA7I. This lack of reactivity of mice immunized against type I strain, may be associated with the immunization protocol. The objective of these experimental infections was to obtain serum samples positive for different *Toxoplasma* strains. Type I strains are highly virulent to mice and lead to animal death in approximately 48 hours. This short period of time does not allow antibody production. To assure mice survival, animals were not infected with tachyzoites of *Toxoplasma* strain RH, but were immunized with RH antigenic suspension. Mice seroconvert, but the induced antibodies did not recognize the selected antigens. Peptide GRA7III was recognized by mice infected with strains type III. Peptides GRA6I/III and GRA7III cross-reacted with some serum samples from mice infected with type II and non-archetypal strains (EHVY for GRA6 and LQEG for GRA7). Since mouse antibodies were specific against each infecting strain, the cross-reactivity found for some sera may reflect specificity problems of these peptides. However, the specificity of these two peptides is higher than 90%.

Serotyping results with peptides specific for the archetypal lineages I, II and III demonstrate that peptide GRA7I is not suitable to distinguish GRA7 type I strains in human sera. However, the serum samples from type I infections were consecutive sera from two patients with a particular way of infection (accidental laboratorial infection with RH strain), which is a limited sample. Natural infections in humans are normally caused by oral ingestion of cysts or oocysts. The two patients infected with the strain RH were infected by inoculation

of tachyzoites. The different immune response induced by these two ways of infection may explain the lack of reactivity of these sera. To confirm this hypothesis, other serum samples from different patients naturally infected with type I strains must be studied. Peptides GRA6II, GRA6I/III and GRA7III were able to recognize serum samples from patients infected with GRA6 type II, GRA6 type I or III and GRA7 type III strains respectively. However, three patients infected with strains type II (LGE-01-4, LGE-01-1 and LGE-2005-FRA) and one patient infected with a type III strain (LGE-97-3) cross-reacted with other peptides in an unspecific manner. Contrary to mouse infections, where these cross-reactions were related with specificity problems, in humans a natural mix infection with more than one strain can not be excluded, although only one strain was isolated by bioassay. Bioassay, compared to direct genotyping on pathological product, was already suspected to select only one strain in case of mixed infection (Villena *et al*., 2004). Although, the peptides tested in this study were not the same as those used by Kong and colleagues (2003), similar results were obtained. In the study performed by Kong and colleagues (2003), only peptides specific for GRA6 type II, GRA6 type I or III and GRA7 type III strains were recognized by most studied serum samples.

Serum samples from twelve patients infected with atypical strains cross-reacted with these peptides, leading to a misclassification of these atypical strains. These atypical strains shared at the GRA6 C-terminal region some polymorphisms with the archetypal strains and at the GRA7 C-terminal region shared the same polymorphisms with GRA7 type I strains. The different reactivity profiles induced by the same strain, or by strains with the same polymorphisms in different patients suggest that different patients develop specific immune responses to the same antigen.

Three peptides specific for atypical strains were tested against serum samples from patients infected with type I, II, III and atypical strains. From these three peptides specific for atypical strains, only peptide Am7 (AETSN) showed a high specificity, since it did not react with any human sera from type I, II or III infections. However, this peptide also has a very low sensitivity, since it was only recognized by 3 out of 16 serum samples from known atypical strains with the same polymorphism (AETSN). Peptides Am6 and Af6 cross-reacted with serum samples infected with archetypal strains. The high rate of reactivity of these two peptides confirms that the region between aa positions 198 and 230 is an immunogenic

epitope, as predicted by the three parameters analysed. However, the increase of size of the single sequence of the peptides seems to diminish the peptide specificity, probably associated with the increase of non-polymorphic aa, since these peptides were also recognized by 80% sera from type I, II or III infections.

4.4 *Toxoplasma gondii* serotypes in humans

Results obtained with previously genotyped strains showed that the selected peptides presented sensitivity and specificity problems, which can limit their used in serotyping. This was especially true for non-archetypal peptides. Peptides Am6 and AF6 had a very low specificity and peptide Am7 a very low sensitivity. Serotyping was used to characterize circulating strains in nature in three continents (Europe, Africa and Latin America). The results obtained in the validation assays, suggest that non-archetypal peptides are less specific than archetypal peptides. Based on that, serotype was defined by the results obtained only with the archetypal peptides. Serum samples reactive only against the atypical peptides were considered with not determined (ND) serotype. *Toxoplasma* strains circulating in Europe belong to the clonal lineages named I, II and III (Ajzenberg *et al*., 2002a; Howe and Sibley, 1995). Type II predominates in France (Ajzenberg *et al*., 2002b; Dumètre *et al*., 2006). In Portugal, the three genotypes have already been described (Canada *et al*., 2002; de Sousa *et al*., 2006; Dubey *et al*., 2006e), type II being the most frequently found. In our study, serotype II was the most prevalent in European serum samples from severe and asymptomatic infections. Similar results were obtained with samples from humans with congenital toxoplasmosis in Poland (Nowakowska *et al*., 2006), from patients with toxoplasmic chorioretinitis from Switzerland, from HIV patients with pulmonary or cerebral toxoplasmosis from France (Morisset *et al*., 2008) and from chronically infected pregnant women from France, Italy and Denmark (Morisset *et al*., 2008; Peyron *et al*., 2006). However, in the present study, a higher prevalence of serotype III, GRA6I/III and mixed serotypes was found among Portuguese patients compared to French patients, which could suggest a different epidemiological pattern of strains circulating in these two countries. This observation and published data from different European countries suggest that genotype II circulates across the continent, with high prevalence in central and north countries. In the south, genetic diversity seems to be higher, with genotype I and III being found. Genotype III was found to be prevalent in Crete and Cyprus in human infections (Messaritakis *et al*., 2008),

while in Spain genotype I, determined by a monolocus *SAG2* typing, was associated with 75% of congenital infections (Fuentes *et al.*, 2004b).

Little information exists about *T. gondii* strains circulating in Africa. *SAG2* type III has been the genotype most frequently described in isolates from chickens from several African countries (Dubey *et al.*, 2003; Dubey *et al.*, 2005a). A multilocus genotyping study of isolates from Ugandan chickens revealed either type I, II or III (Lindström *et al.*, 2008). The few strains isolated from patients from West and Central Africa exhibited a mixture of type I and III alleles, when studied by a multilocus microsatellite analysis (Ajzenberg *et al.*, 2005). In the present study, GRA6I/III profile was the most prevalent being recognized by 19.8% (29.8% if we exclude the serum samples that did not react with the peptides) of the African serum samples. This suggests that GRA6 serotypes I and III are prevalent in Africa. Serotype I (reaction with GRA7I or GRA6I/III and GRA7I) and III (reaction with GRA7III or GRA6I/III and GRA7III) were also found for African patients, but with a low prevalence (similar to prevalence of serotype II). Peptides GRA7I and GRA7III, specific for these two serotypes had a lower sensitivity compared with peptide GRA6I/III, which may explain the low prevalence of these serotypes. If peptides GRA7I and GRA7III had a higher sensitivity, more sera with GRA6I/III profile could be differentiated in serotype I or III.

In South America, atypical genotypes are mainly found (Ajzenberg *et al.*, 2004; Ferreira *et al.*, 2006; Khan *et al.*, 2007). Eleven cases of toxoplasmosis were reported in a village from Suriname; at least 5 isolates corresponded to only one non-archetypal strain (Demar *et al.*, 2007). In French Guiana, most of the reported cases of disseminated toxoplasmosis in immunocompetent patients were also associated with non-archetypal strains (Ajzenberg *et al.*, 2004; Carme *et al.*, 2002). Considering this, it should be expected that sera from these cases would not react with peptides specific for the archetypal strains, since these non-archetypal strains possess specific polymorphisms, distinguishing them from Type I, II and III strains. Of serum samples from Latin American countries, GRA6I/III profile was the most prevalent, being found in 30.6% (39.3% of successfully serotyped sera) of the patients. Serotypes I (reaction with GRA7I or GRA7I and GRA6I/III) and III (reaction with GRA7III or GRA7III and GRA6I/III) were also found. Peyron and colleagues (2006) and Morisset and colleagues (2008) described similar results with serotyping of chronically infected pregnant women from Colombia and patients with ocular and multivisceral acute infections from

Brazil, Colombia and French Guiana, where a type I and III profile was found. The prevalence of serotype I and III in these countries may be explained by the peptides used for serotyping. In both studies, serotyping was based on GRA5 and GRA6 peptides specific for the archetypal lineages I, II and III. These peptides may have cross-reacted with some atypical strains leading to a misclassification of the atypical strains as type I and III. In the present work, cross-reaction with GRA6 peptides was described and demonstrated in atypical strains from French Guiana and Suriname. In the present study, a type II profile was found in 4.5% of patients from Latin America. Among these, a serum samples from one patient from Colombia only recognized peptide GRA6 type II, indicating a possible type II infection. This serotype was not found in pregnant women from Colombia (Peyron *et al*., 2006).

Most studies conducted in South American countries refer the absence of genotype II in that region. Serotyping of patients from Uruguay reveals the existence of serotype II. Interestingly, this serotype has not a homogeneous distribution around the country. The prevalence of serotype II seems to increase in a north-south direction, while mixed profiles are more prevalent in the North, close to the Brazilian border. These mixed profiles may indicate the existence of atypical genotypes, as was demonstrate with serotyping of serum samples from infections with atypical strains from Suriname and French Guiana. Atypical genotypes were found among chickens from Rio Grande do Sul (the Brazilian state close to the Uruguay border) having alleles I and III for GRA6 (Dubey *et al*., 2007e). The presence of serotype II in the South of Uruguay nearer to Montevideo area may reflect the import of type II strains in this region with many trade exchanges with Europe.

The limited number of sera from different geographical origin and limitations inherent to the serotyping method (limited number of peptides from only two loci, cross-reactivity and lack of sensitivity) are two important biases in interpretation of relationship between serotype and clinical disease. To better understand the hypothetical association between serotype and clinical disease, serum samples from patients with a specific pathology from different geographical regions must be studied by a large number of discriminative peptides. However, the present results suggest that the same type is responsible for symptomatic and asymptomatic infections and that the type is determined by the geographical origin of the infection. Similar conclusion was achieved by Morisset and colleagues (2008), with patients with different pathologies from Europe and South America. In Europe, a homogeneous

distribution of serotype II was found independently of the associated disease, while in South America a homogeneous distribution of serotypes I and III were found.

Validation assays with the atypical peptides (Am6, Am7 and Af6) showed that these peptides have sensitivity and specificity problems, which limits their use in serotyping assays. Serum samples with unknown genotype from the three studied continents reacted with these peptides. However, reactivity was clearly higher for South America and African countries then for European countries. Peptide Am7, as already observed in the validation assay only reacted with serum samples from Ivory Coast and Uruguay, which is in agreement with its high specificity. Reaction of peptides Am6 and Af6 with serum samples from Europe may reflect the lack of specificity of these two peptides.

4.5 Limits of serotyping

Besides specificity problems, another limitation of serotyping is sensitivity. Some infections can not be serotyped. A considerable number of samples did not recognize the studied peptides. This was actually the case for immunocompetent asymptomatic patients from Europe and Africa serotyped with our GRA6 and GRA7 archetypal peptides, where 37.7% and 36.8% respectively of the studied serum samples were not serotyped. Serotyping with a larger number of peptides, specific for both archetypal and non-archetypal strains increases the sensitivity of the method and consequently diminishes the number of untyped samples, but the low specificity of these non archetypal peptides does not allow drawing any conclusion about infecting genotype. For congenital infections and other *T. gondii* related diseases (cerebral, pulmonary and ocular) from Europe the number of non-serotyped infections was 20.5% and 28.6% respectively. Similar results were obtained for congenital infections from Poland (15.4%) by Nowakowska and colleagues (2006) and for cerebral and ocular toxoplasmosis from North America (29.2%) by Kong and colleagues (2003). It seems pertinent to link the IgG isotypes kinetic (antibody profile) with the time of infection. The kinetics of the humoral response might explain the high rate of non-serotyped asymptomatic chronic infections especially in Europe. Determination of the specific isotype present at each infection stage may be important for the study of the unserotyped infections. However, it should be noted that in mice there was a homogeneous recognition of the isotype IgG2a independently of the time of infection. The study of this isotype does not increase assay

sensitivity. The lack of sensitivity may also be related to the established protocol. Synthetic peptides directly attached to special plates, seems to be less efficient than coupling to carrier proteins. The number of serum samples tested in this study was considerable high, when compared with other serotyping studies. This fact may explain the higher number of non reactive sera obtained in the present work. The preservation of the sera is essential to assure quality results. Many of the studied sera were old and have been previously used in diagnostic assays. Serum samples from Uruguay were the most recently collected and were in perfect conditions. The number of non reactive sera was only 14.4% among 208 patients from Uruguay. Published data regarding the use of recombinant proteins derived from GRA6 and GRA7 showed that these proteins do not have a sensitivity of 100% in human sera ELISA. The immune response to GRA6 is very heterogenic (Ferrandiz *et al* 2004). The sensitivity found by this author for rGRA6-ELISA was 83%. Lecordie and colleagues (2000) studied two recombinant proteins derived from GRA6. The recombinant protein with the GRA6 C-terminal showed only a sensitivity of 10% by ELISA, while the GRA6 N-terminal fusion protein showed a sensitivity of 96%. Studies with GRA7 recombinant proteins showed a sensitivity of 81% (Jacobs *et al*. 1999). In congenital infected children sensitivity of rGRA7 proteins is lower. For children with less than 4 months, sensitivity was 64%, while for older children sensitivity was only 11% (Altcheh *et al*., 2006). These two antigens are considered characteristic of the acute phase of infection (Gatkowska *et al*., 2006; Pfrepper *et al*., 2005; Redlich and Muller, 1998). Consequently, these two antigens have a higher sensitivity with serum samples from acute infections. In this study, sensitivity of serotyping was 79.5% for European patients with congenital infection, while for immunocompetent asymptomatic patients was 62.3%. Congenital infections are usually associated with a seroconversion during pregnancy, i.e. a recently acquired infection, while asymptomatic patients have chronic infections. Serum samples from congenitally infected children seem to be more sensible for serotyping assays than mother sera. Fourteen (73.7%) out of nineteen children sera were correctly serotyped, while eleven (52.4%) out of twenty-one mother's sera were correctly serotyped.

4.6 Serotyping of naturally infected animals

Serotyping of serum samples from domestic animals was here reported for the first time. The strains used to validate the protocol for serotyping of animal sera were isolated (by

bioassay in mice) of tissues from chickens and pigs seropositive for *T. gondii* (de Sousa *et al*., 2006; Dubey *et al*., 2006e). From peptides specific for archetypal strains, peptide GRA6II gave the best results, being specifically recognized by type II strains from chickens and pigs, but with low sensitivity (52.6%). Peptide GRA6I/III also has a low sensitivity (only one out of seven serum samples from chickens and pigs infected with type III strains were recognized). This peptide was not very specific for serum samples from pigs. Peptide GRA7III does not recognize any serum sample from animals infected with type III strains. The match rate between genotyping and serotyping using the archetypal strains-specific peptides (GRA6II, GRA6I/III, GRA7I and GRA/III) was 36.4% for chickens and only 13.3% for pigs. Two chickens and four pigs infected with type II strains cross-reacted with other peptides. Like for human infections, these cross-reactions in naturally infected animals may reflect the existence of a mix infection, since only one strain is isolated by bioassay. The number of non reactive sera was high, both in chickens and pigs. The high rate of cross-reactions and the low sensitivity of this method, limits the use of the selected peptides for serotyping natural infections in animals, especially in pigs. Peptides specific for atypical strains (Am6, Af6, Am7) reacted with type II and III animal sera. These data demonstrates that serotyping based on the studied peptides presents some limitations for typing strains of animal origin. In both studies, isolates were not obtained from all bioassayed samples. For those animals, serotyping was used in an attempt to determine the genotypes from the infected animals for which no isolates were obtained. Serotyping of these untyped infections suggests that for chickens and pig, serotype II prevails (25.7% and 20.7% respectively), which is in agreement with the results obtained by genotyping of *T. gondii* isolates from these animals, while for sheep serotype III seems to be more prevalent (20%). Sheep and pigs have the same geographic origin (Northeast of Portugal), while chickens came from the Northwest. The fact that the same serotype was found in different species from different places and that different serotypes were found in the same geographic area for different species suggests that serotype selection was not made by geographic region. The existence of different serotypes among animal species (pigs and sheep) in the same geographic environment, suggests the existence of a strain selection by the host. Although, serotype III was the most frequently found among sheep, studies in Spain and France described the presence of genotype II in sheep (Dumètre *et al*., 2006; Fuentes *et al*., 2004a). However, genotype III seems to be more frequent in countries from South Europe (Messaritakis *et al*., 2008). The results obtained in the present

study re-inforce this hypothesis, since serotype III was also more frequent among Portuguese patients when compared with French patients.

Conclusions

GRA6 was already described as a highly polymorphic locus. Our results confirm the high rate of polymorphisms of this locus. *GRA7* is also a locus with a considerable number of polymorphisms, which could be interesting for genotyping methods. The apparent limited rate of polymorphisms in GRA8 peptide does not make this antigen suitable for serotyping. GRA6 peptide is very discriminative, with polymorphisms capable of differentiating the type II from type I and III strains as well as several non-archetypal strains. GRA7 peptide has not the same discriminative power than GRA6, but it can also differentiate the three archetypal lineages and some non-archetypal strains. Peptides to be used in serotyping must be polymorphic and immunogenic, which limits the peptides to specific regions of the protein. Three-fold repetition of the sequence in a single peptide is a valuable approach for serotyping, since it allows amplifying the reaction. Infection due to strains belonging to the archetypal lineages I, II and III can be serotyped with the peptides GRA6II, GRA6I/III and GRA7III. Peptides specific for non-archetypal strains have a poor specificity. Peptide Am7 was the most specific, being exclusively recognized by atypical strains. However, this peptide has a low sensitivity. In Europe the prevalent serotype is type II, while in Africa and Latin America, serotype GRA6I/III prevails. It was also demonstrated the circulation of serotype II in South America. These results suggest that the serotype is determined by the geographical origin of the infection. According to geographic origin, the same serotype is responsible for symptomatic and asymptomatic infections. The studied peptides present some limitations for genotyping *T. gondii* strains from domestic animals.

Perspectives

The present work revealed several limitations of serotyping as a valuable method for typing *T. gondii* strains. At least, in the performed ELISA test using as antigens the strain-specific synthetic peptides described. First, the limited number of studied loci (only two), which raised the same problems found in single locus genotyping. Based on this, the first step for future studies must be the selection of other polymorphic antigens. New antigens have been described from GRA and ROP proteins that if immunogenic and polymorphic could be interesting to new peptides definition. Recently, peptides derived from ROP18 were identified as useful in strain identification, and possibly mouse virulence prediction. Peptides should be defined for the three clonal lineages and for the non-archetypal lineages as well. For each lineage, peptides must be selected from the highest number of antigens as possible. Only using several peptides from different markers for each strain type will be possible to validate serotyping as a useful typing method. In order to establish the most efficient protocol, peptides should be tested by the three different published protocols. A different protocol based on peptide microarrays should also be explored, in order to develop a multilocus serotyping method. These three steps will be crucial to define the future of serotyping and its utility as a typing method. In the perspective of these objectives being achieved, serotyping will become a typing method with high potential. Being a non-invasive method, serotyping can be easily applied in large scale epidemiological studies. These studies conducted in wild and domestic animals from different continents are important to understand if a strain selection is made by the host and the geographical genotype distribution. Concerning human infection these epidemiological studies may clarify if a relationship can be established between strain genotype and human disease. The development of multiple peptides from different antigens for each genotype will be essential to understand the extension of mixed infections with different genotypes. A multilocus serotyping will allow distinguishing non-archetypal serotypes similarly to a multilocus genotyping. The accomplishment of these objectives will probably have impact on the diagnosis and follow up of infected patients. Serotyping may be useful in the detection of a re-infection with a different strain, which may be particularly relevant in the follow up of pregnant women. Toxoplasmosis being a

foodborne disease, serotyping may become a powerful tool for the detection of *T. gondii* infections by non-archetypal strains in animals for human consumption.

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Appendixes

Appendix 1 - *Toxoplasma gondii* seroprevalence

Table 1: *Toxoplasma gondii* seroprevalence in wild animals with different geographic origin

Geographic origin	Host	Assay*	Sample	Seroprevalence (%)	Reference
Spain	Fallow deer	MAT	79	24	Gauss *et al.*, 2006
	Red deer	MAT	441	15.6	
	Roe deer	MAT	278	39.2	Gamarra *et al.*, 2008
	Rabbits	MAT	456	14.2	Almería *et al.*, 2004
Czech Republic	Red deer	IFAT	377	45	Bartova *et al.*, 2007
	Fallow deer	IFAT	143	17	
	Mouflon	IFAT	105	9	
Svalbard	Arctic fox	MAT	594	43	Prestrud *et al.*, 2007
	Barnacle geese	MAT	149	7	
United Kingdom	Red fox	IFAT	549	20	Hamilton *et al.*, 2005
USA	Sows	MAT	2617	15.1	Dubey *et al.*, 1995
	Raccoon	MAT	188	67	
	Opossum	MAT	128	22.7	
	House mouse	MAT	1243	2.1	

*MAT – modified agglutination test; ELISA - enzyme lynked immunosorbent assay; IFAT – immunofluorescence antibody test

Table 2: *Toxoplasma gondii* seroprevalence in domestic animals with different geographic origin

Geographic origin	Host	Assay*	Sample	Seroprevalence (%)	Reference
Portugal	Domestic cat	MAT	204	35.8	Lopes *et al*., 2008
France	Lamb	MAT	164	22.0	Dumètre *et al*., 2006
	Ewe		93	65.6	
Spain	Cat	IFAT	585	32.3	Miró *et al*., 2004
Germany	Pig	ELISA/IFAT	230	18.5 / 16.5	Damriyasa *et al*., 2004
USA	Lamb	MAT	383	27.1	Dubey *et al*., 2008a
	Pig	MAT	4252	2.3	Dubey *et al*., 1995
	Cat	MAT	391	68.3	
Mexico	Chicken	MAT	208	6.2	Dubey *et al*., 2004a
	Cat	MAT	105	21	Alvarado-Esquivel *et al*., 2007
	Dog	MAT	101	51.5	Dubey *et al*., 2007a
Costa Rica	Chicken	MAT	144	40.1	Dubey *et al*., 2006c
Panama	Pig	IFAT	290	32.1	Correa *et al*., 2008
Colombia	Dog	MAT	309	16.8	Dubey *et al*., 2007b
Brazil	Cat	MAT	237	35.4	Pena *et al*., 2006
	Chicken	MAT	198	65.2	da Silva *et al*., 2003

Geographic origin	Host	Assay*	Sample	Seroprevalence (%)	Reference
Colombia	Dog	MAT	309	16.8	Dubey *et al*., 2007b
Brazil	Cat	MAT	237	35.4	Pena *et al*., 2006
	Chicken	MAT	198	65.2	da Silva *et al*., 2003
Sri Lanka	Chicken	MAT	100	39	Dubey *et al*., 2005b
Israel	Cat	ELISA	1062	16.8	Salant and Spira, 2004
	Chicken	MAT	96	46.6	Dubey *et al*., 2004b
South Africa	Sheep	IFAT/ELISA	600	5.6 / 4.3	Samra *et al*., 2007
Egypt	Chicken	MAT	121	40.4	Dubey *et al*., 2003
Morocco	Sheep	ELISA	261	27.6	Sawadogo *et al*., 2005

*MAT – modified agglutination test; ELISA – enzyme lynked immunosorbent assay; IFAT – immunofluorescence antibody test

Appendix 2 – *Toxoplasma gondii* strains

Strain	Host	Geographical origin	Genotype	Allele				
				TUB2	*W35*	*TgM-A*	*B18*	*B17*
RH	Human	USA	I	1	1	1	1.3	1
BEVERLEY*	Rabbit	England	II	2.3	2	2	2	2.3
NED*	Human	France	III	2.3	3	3	1.3	2.3
TgCkCo24	Cat fed by chicken pool	Colombia	Variant of I	1	1	1	1.3	2.3
GANGI*	Human	Africa	Variant of II	2.3	2	1	2	2.3
ENVL-2002-MAC*	Monkey	Barbados	Variant of III	1	3	3	1.3	2.3
CRL-2004-MOT	Human	Cameroon	III/I	1	1	3	1.3	2.3
IPB-2003-COG	Human	Belgium	II/III	2.3	2	3	2	2.3
TgCkBr10	Chicken	Brazil	Atypical	1	2.3	2	4	1
TgCkBr11	Chicken	Brazil	Atypical	1	2.3	3	4	4
TgCkBr25	Chicken	Brazil	Atypical	1	2.3	3	4	14
TgCkBr4	Chicken	Brazil	Atypical	1	2.3	3	1.3	9
RMS-1994-LEF	Human	France	Atypical	1	2	3	1.3	10
TgCkBr3	Chicken	Brazil	Atypical	2.3	2.3	4	8	14
TgCkBr22	Chicken	Brazil	Atypical	2.3	2.3	4	8	1
TgCkBr6	Chicken	Brazil	Atypical	1	2.3	2	9	4
TgCkBr7	Chicken	Brazil	Atypical	1	2.3	2	9	4
TgCkAr23	Cat fed by chicken pool	Argentina	Atypical	4	3	2	2	7
IPP-2002-URB*	Human	France	Atypical	4	2	2	1.3	7
GUY-2003-BAS*	Human	French Guiana	Atypical	2.3	2	4	5	5
GUY-2004-TER	Human	French Guiana	Atypical	2.3	9	4	2	4
TgCkBr1	Chicken	Brazil	Atypical	2.3	2.3	2	1.3	14

Strain	Host	Geographical origin	Genotype	Allele				
				TUB2	*W35*	*TgM-A*	*B18*	*B17*
TgCkBr18	Chicken	Brazil	Atypical	2.3	2.3	2	1.3	14
CCH-2005-REN	Human	Guadeloupe	Atypical	1	3	3	4	1
IPP-2002-BAT*	Human	French Guiana	Atypical	1	3	3	4	2.3
P80*	Pig	USA	Atypical	1	3	3	2	6
GUY-2004-LAB	Human	French Guiana	Atypical	1	2	4	1.3	12
GUY-2002-MAT*	Human	French Guiana	Atypical	1	7	4	1.3	11
GUY-2001-DOS*	Human	French Guiana	Atypical	2.3	4	4	1.3	5
GUY-2002-KOE*	Human	French Guiana	Atypical	2.3	4	4	5	4
GUY-2004-AKO	Human	French Guiana	Atypical	1	2.3	4	1.3	4
MAR-2000-HOU	Human	Brazil	Atypical	2.3	2	3	1.3	1
TgCkBr24	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	4
TgCkBr21	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	15
TgCkBr19	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	15
TgCkBr17	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	4
TgCkBr15	Chicken	Brazil	Atypical	2.3	2.3	3	4	1
TgCkBr14	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	6
TgCkBr12	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	4
TgCkBr5	Chicken	Brazil	Atypical	1	1	3	1.3	1
TgCkBr9	Chicken	Brazil	Atypical	1	1	3	1.3	1
TgCkBr23	Chicken	Brazil	Atypical	1	1	3	1.3	14
CCH-2004-NIA	Human	Senegal	Atypical	2.3	1	3	1.3	2.3
PSP-2003-KOM*	Human	Cameroon	Atypical	1	6	3	1.3	1

Strain	Host	Geographical origin	Genotype	Allele				
				TUB2	*W35*	*TgM-A*	*B18*	*B17*
RMS-2003-DJO	Human	Benin	Atypical	1	1	3	1.3	1
WIK*	Human	Africa	Atypical	1	1	3	1.3	1
P89	Pig	USA	Atypical	1	2.3	3	1.3	6
GUY-2003-MEL*	Human	French Guiana	Atypical	2.3	2	4	2	5
TgCkBr16	Chicken	Brazil	Atypical	2.3	2.3	3	1.3	4
TOU-2002-ALI*	Human	Reunion Island	Atypical	2.3	2	3	1.3	4
TgCkBr2	Chicken	Brazil	Atypical	1	2.3	2	1.3	4
GUY-2004-JAG	Jaguar	French Guiana	Atypical	2.3	2	4	1.3	2.3

Alleles 1, 2, 3 are reserved for clonal lineages I, II and III. Allele 1.2 means that types I and II share the allele; allele 2.3 means that types II and III share the allele; allele 1.3 means that types I and III share the allele. Allele 4 and above are used for atypical alleles.

* Strains already published (Ajzenberg *et al*., 2004)

Appendix 3 – Sequencing results

Table 1: Haplotypes defined for *Toxoplasma gondii* strains

Strain*	Host	Geographical origin	Genotype	Haplotype		
				GRA6	GRA7	GRA8
RH	Human	USA	I	1	1	1
TgCkCo24	Cat fed by chicken pool	Colombia	Variant of I	1	1	ND
TgCkBr5	Chicken	Brazil	Atypical	11	1	ND
TgCkBr9	Chicken	Brazil	Atypical	11	1	1
TgCkBr23	Chicken	Brazil	Atypical	11	1	ND
CCH-2004-NIA	Human	Senegal	Atypical	11	1	ND
PSP-2003-KOM	Human	Cameroon	Atypical	11	1	1
RMS-2003-DJO	Human	Benin	Atypical	11	1	ND
WIK	Human	Africa	Atypical	11	1	1
GUY-2003-MEL	Human	French Guiana	Atypical	13	1	ND
BEVERLEY	Rabbit	England	II	2	2	ND
GANGI	Human	Africa	Variant of II	2	2	ND
TgCkBr10	Chicken	Brazil	Atypical	2	4	ND
TgCkBr11	Chicken	Brazil	Atypical	2	4	ND
TgCkBr25	Chicken	Brazil	Atypical	2	4	ND
TgCkBr4	Chicken	Brazil	Atypical	4	4	2
RMS-1994-LEF	Human	France	Atypical	4	4	2
TgCkAr23	Cat fed by chicken pool	Argentina	Atypical	5	7	2
IPP-2002-URB	Human	France	Atypical	5	8	ND
TgCkBr2	Chicken	Brazil	Atypical	19	4	ND
TgCkBr1	Chicken	Brazil	Atypical	7	4	5

Strain*	Host	Geographical origin	Genotype	Haplotype		
				GRA6	GRA7	GRA8
TgCkBr18	Chicken	Brazil	Atypical	7	4	5
CCH-2005-REN	Human	Guadeloupe	Atypical	7	1	ND
IPP-2002-BAT	Human	French Guiana	Atypical	7	ND	ND
P80	Pig	USA	Atypical	7	ND	ND
TgCkBr16	Chicken	Brazil	Atypical	18	4	ND
MAR-2000-HOU	Human	Brazil	Atypical	10	4	1
TgCkBr24	Chicken	Brazil	Atypical	10	4	ND
TgCkBr17	Chicken	Brazil	Atypical	10	4	ND
TgCkBr15	Chicken	Brazil	Atypical	10	1	ND
TgCkBr14	Chicken	Brazil	Atypical	10	11	ND
TgCkBr12	Chicken	Brazil	Atypical	10	12	ND
TgCkBr21	Chicken	Brazil	Atypical	10	3	ND
TgCkBr19	Chicken	Brazil	Atypical	10	3	ND
NED	Human	France	III	3	3	1
ENVL-2002-MAC	Monkey	Barbados	Variant of III	3	3	ND
CRL-2004-MOT	Human	Cameroon	III/I	3	1	ND
IPB-2003-COG	Human	Belgium	II/III	3	2	ND
TgCkBr3	Chicken	Brazil	Atypical	16	5	1
TgCkBr22	Chicken	Brazil	Atypical	16	5	ND
TgCkBr6	Chicken	Brazil	Atypical	17	6	5
TgCkBr7	Chicken	Brazil	Atypical	17	6	ND
TOU-2002-ALI	Human	Reunion Island	Atypical	14	6	ND
GUY-2004-TER	Human	French Guiana	Atypical	6	6	1
GUY-2003-BAS	Human	French Guiana	Atypical	6	9	ND

Strain*	Host	Geographical origin	Genotype	Haplotype		
				GRA6	GRA7	GRA8
GUY-2004-LAB	Human	French Guiana	Atypical	8	9	1
GUY-2002-MAT	Human	French Guiana	Atypical	8	9	1
GUY-2001-DOS	Human	French Guiana	Atypical	9	9	1
GUY-2002-KOE	Human	French Guiana	Atypical	9	10	3
GUY-2004-AKO	Human	French Guiana	Atypical	10	9	3
P89	Pig	USA	Atypical	12	13	ND
GUY-2004-JAG	Jaguar	French Guiana	Atypical	15	14	4

*GenBank accession numbers – appendix 4

∴ ND – not determined

Table 2: *GRA6* nucleotide sequences for the 49 atypical strains of *Toxoplasma gondii*. Periods (.) indicate nucleotide identical to haplotype 1 and dashes (-) indicate deletion.

Haplotype	Positions of variable nucleotides																																		
	41	57	71	95	106	146	162	167	170	171	293-296	304	336	403	412	418	561	576	579	591	598	614	635-649	658-660	669	671	674	677	690	692	693	702	711			
1	C	G	G	G	C	G	G	G	A	A	---A	A	T	C	A	C	A	A	G	A	C	G	TACGGAGGCAGAGGT	AGC	A	A	C	C	A	C	G	T	A			
2	T	.	T	.	.	.	.	.	.	G	....	T	C	.	.	.	.	G	.	.	.	.	----------	---	.	.	.	.	G	A	.	A	T			
4	T	.	T	.	.	.	.	.	.	.	....	T	C	.	.	.	.	G	.	.	.	.	.....	---	G	.	G	.	G	A	.	A	T			
19	T	.	T	.	.	.	.	T	.	G	....	T	C	.	.	.	.	.	.	G	.	.	.....	---	.	.	.	.	.	.	.	A	.			
15	T	.	T	A	.	T	.	.	.	G	GTAG	T	C	.	.	T	.	G	.	.	.	.	G.....	..G	.	.	.	.	.	.	.	A	.			
3	.	.	T	.	T	.	A	.	.	.	....	T	.	.	.	.	C	.	.	.	.	C	.....	..G	.	.	.	G	.	.	.	.	.			
16	.	.	T	.	.	.	.	.	.	.	....	T	.	.	.	.	C	.	.	.	.	C	.....	..G	.	.	.	G	.	.	A	.	.			
10	.	.	T	.	.	.	.	.	.	.	....	T	.	T	G	.	C	.	A	.	.	.	...A.....	..G	.	.	.	G	.	.	A	.	.			
18	.	.	T	.	.	.	.	.	.	.	....	T	.	.	G	.	C	.	A	.	.	.	...A.....	..G	.	.	.	G	.	.	A	.	.			
17	.	.	T	.	.	.	A	.	.	.	....	T	.	.	.	.	C	.	.	.	A	.	...A.....	..G	.	.	.	G	.	A	.	.	.			
5	.	.	T	.	.	.	A	.	.	.	....	T	.	.	.	.	C	.	.	.	.	.	...A.....	..G	.	.	.	G	G	A	.	.	.			
6	.	.	T	.	.	.	A	.	.	.	...G	T	.	.	.	.	C	.	A	.	.	.	...A.....	..G	.	.	.	G	G	A	.	.	.			
7	.	.	T	.	.	.	A	.	.	.	...G	T	.	.	.	.	C	.	A	.	.	.	----------	..G	.	.	.	G	G	A	.	.	.			
14	.	.	T	.	.	.	A	.	.	.	...G	T	.	.	.	.	C	.	A	.	.	.	...A.....	..G	.	.	.	.	G	A	.	.	.			
8	.	A	T	.	.	.	.	.	.	.	....	T	.	.	.	.	C	.	A	.	.	.	...A.....	..G	.	.	.	G	.	.	.	.	.			
9	.	.	T	.	.	.	.	.	.	.	....	T	.	.	.	.	C	.	A	.	.	.	...A.....	..G	.	.	.	G	.	.	.	.	.			
11	.	.	T	.	.	.	.	.	C	.	....	T	.	.	.	.	C	.	.	.	.	.	...A.....	..G	.	G	.	G	.	.	.	.	.			
12	.	.	T	.	.	.	.	.	C	.	....	T	.	.	.	.	C	.	.	.	.	.	...A.....	---	.	G	.	G	.	.	.	.	.			
13	.	.	T	.	.	.	.	.	.	.	....	T	.	.	.	.	C	.	A	.	.	.	...A.....	..G	.	G	.	G	.	.	.	.	.			

Table 3: GRA6 amino acid sequences. Periods (.) indicate amino acid identical to haplotype 1 and dashes (-) indicate deletion. Haplotype 1 is found for type I as well as a variant of I. Haplotype 2 is found for type II as well as variant of II and atypical. Haplotype 3 is found for type III as well as variant of III.

Haplotypes	Positions of variable amino acids																								
	7	12	17	25	42	47	49	50	91	92	94	105	180	185	186	190	198	204-208	213	216-219	223	224	227	230
1 (Type I)	H	R	V	V	V	G	V	K	–	T	E	V	Q	D	G	D	G	GYGGR	A	DRRP	E	R	V	Y
2 (Type II)	Y	.	L	.	.	.	.	R	–	.	D	A	.	G	.	.	.	–––––	–	. . . .	G	S	E	F
4	Y	.	L	.	.	.	.	.	–	.	D	A	.	G	.	.	.	. . . . .	–	G . G .	G	S	E	F
19	Y	.	L	.	.	.	F	R	–	.	D	A	.	.	.	G	.	. . . . .	–	. . . .	.	.	E	.
15	Y	.	L	I	F	.	.	R	D	A	D	A	.	G	.	.	.	. D . . .	G	. . . .	.	.	E	.
3 (Type III)	.	.	L	.	.	D	.	.	–	.	D	.	P	.	.	.	R	. . . . .	G	. . . A	.	.	.	.
16	.	.	L	.	.	.	.	.	–	.	D	.	P	.	.	.	R	. . . . .	G	. . . A	.	H	.	.
10 and 18	.	.	L	.	.	.	.	.	–	.	D	.	P	.	D	.	.	. . R . .	G	. . . A	.	H	.	.
17	.	.	L	.	.	D	.	.	–	.	D	.	P	.	.	.	.	. . R . .	G	. . . A	.	S	.	.
5	.	.	L	.	.	D	.	.	–	.	D	.	P	.	.	.	.	. . R . .	G	. . . A	G	S	.	.
6	.	.	L	.	.	D	.	.	–	A	D	.	P	.	D	.	.	. . R . .	G	. . . A	G	S	.	.
7	.	.	L	.	.	D	.	.	–	A	D	.	P	.	D	.	.	–––––	G	. . . A	G	S	.	.
14	.	.	L	.	.	D	.	.	–	A	D	.	P	.	D	.	.	. . R . .	G	. . . .	G	S	.	.
8	.	H	L	.	.	.	.	.	–	.	D	.	P	.	D	.	.	. . R . .	G	. . . A	.	.	.	.
9	.	.	L	.	.	.	.	.	–	.	D	.	P	.	D	.	.	. . R . .	G	. . . A	.	.	.	.
11	.	.	L	.	.	.	.	Q	–	.	D	.	P	.	.	.	.	. . R . .	G	. G . A	.	.	.	.
12	.	.	L	.	.	.	.	Q	–	.	D	.	P	.	.	.	.	. . R . .	–	. G . A	.	.	.	.
13	.	.	L	.	.	.	.	.	–	.	D	.	P	.	D	.	.	. . R . .	G	. G . A	.	.	.	.

Table 4: *GRA7* nucleotide sequences for the 47 atypical strains of *Toxoplasma gondii*. Periods (.) indicate nucleotide identical to haplotype 1.

Haplotype	Positions of variable nucleotides																												
	47	**114**	**137**	**340**	**367**	**382**	**396**	**502**	**505**	**523**	**528**	**532**	**533**	**540**	**550**	**556**	**568**	**569**	**573**	**577**	**605**	**613**	**619**	**625**	**629**	**683**	**690**	**709**	**715**
1 (Type I)	C	A	T	C	A	C	G	C	G	G	A	A	G	C	A	G	G	G	T	T	C	C	A	C	C	T	A	G	G
14	T	.	.	.	.	.	A	A	C	C	.	.	C	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2 (Type II)	T	.	.	.	.	.	A	A	C	C	.	.	C	A	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.
3 (Type III)	T	G	.	G	C	A	A	A	C	C	.	.	C	.	T	.	A	.	.	A	T	.	C	A	A	C	C	.	A
11	T	.	.	G	C	A	A	A	C	C	.	.	C	.	T	.	A	.	.	A	T	.	C	A	A	C	C	.	A
4	T	.	.	G	C	.	A	A	C	C	.	.	C	.	T	.	A	.	.	A	T	.	C	A	A	C	C	.	A
7	T	.	.	G	C	.	A	A	C	C	.	.	C	.	T	.	A	.	G	A	T	.	C	A	A	C	C	.	A
12	T	.	.	G	C	.	A	A	C	C	G	.	C	.	T	.	A	.	.	A	T	.	C	A	A	C	C	.	A
13	.	.	.	G	C	A	A	A	C	C	.	.	C	.	T	.	A	.	.	A	T	.	C	A	A	C	C	.	A
8	T	.	.	G	C	.	A	A	C	C	.	.	C	.	T	.	A	.	G	A	T	T	C	A	A	C	C	.	A
5	T	.	.	.	.	.	A	A	C	C	.	G	C	.	.	A	A	A	.	.	.	.	.	.	.	.	.	.	.
9	T	.	C	.	.	.	A	A	C	C	.	G	C	A	.	A	A	A	.	.	.	.	.	.	.	.	.	.	.
10	T	.	C	.	.	.	A	A	C	C	G	G	C	A	.	A	A	A		.	.	.	.	.	.	.	.	.	.
6	T	.	.	.	.	.	A	G	C	C	.	G	C	A	.	A	A	A	.	.	.	.	.	.	.	.	.	.	.

Table 5: GRA7 amino acid sequences. Periods (.) indicate amino acid identical to haplotype 1. Haplotype 1 is found for type I as well as a variant of I. Haplotype 2 is found for type II as well as variant of II and atypical. Haplotype 3 is found for type III as well as variant of III.

Haplotype	Positions of variable amino acids																						
	8	**38**	**106**	**115**	**120**	**160**	**161**	**167**	**170**	**172**	**176**	**178**	**182**	**185**	**194**	**197**	**199**	**201**	**202**	**220**	**222**	**229**	**231**
1 (Type I)	S	I	R	N	H	L	V	E	R	D	T	G	G	L	A	L	M	L	T	L	Q	E	G
14	F	.	.	.	.	I	L	Q	T	E	.	.	.	.	.	.	.	.	.	.	.	.	.
2 (Type II)	F	.	.	.	.	I	L	Q	T	E	.	.	S	.	.	.	.	.	.	.	.	K	.
3 (Type III), 11	F	.	G	H	N	I	L	Q	T	.	S	.	S	I	V	.	L	I	K	P	H	.	R
4, 7, 12	F	.	G	H	.	I	L	Q	T	.	S	.	S	I	V	.	L	I	K	P	H	.	R
13	.	.	G	H	N	I	L	Q	T	.	S	.	S	I	V	.	L	I	K	P	H	.	R
8	F	.	G	H	.	I	L	Q	T	.	S	.	S	I	V	F	L	I	K	P	H	.	R
5	F	.	.	.	.	I	L	Q	A	.	.	S	N	.	.	.	.	.	.	.	.	.	.
9, 10	F	T	.	.	.	I	L	Q	A	E	.	S	N	.	.	.	.	.	.	.	.	.	.
6	F	.	.	.	.	V	L	Q	A	E	.	S	N	.	.	.	.	.	.	.	.	.	.

Table 6: *GRA8* nucleotide sequences for atypical strains of *Toxoplasma gondii*. Periods (.) indicate identical nucleotide and dashes (-) deletions.

Haplotype	Positions of variable nucleotide																	
	74	**108**	**121**	**128**	**255**	**292**	**315**	**325**	**349**	**414**	**592**	**615**	**628**	**629**	**630**	**665**	**686**	**737**
1	A	A	A	A	C	T	T	G	G	C	G	G	C	A	C	G	T	T
3	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.
4	.	.	.	G	.	.	G	.	.	.	.	C	.	.	.	C	.	.
5	G	T	G	.	T	C	G	C	A	.	A	T	.	.	.	C	G	C
2	.	G	G	.	T	C	G	C	.	.	A	T	-	-	-	C	G	.

Table 7: GRA8 amino acid sequences. Periods (.) indicate identical amino acid and dashes (-) deletions.

Haplotype	Positions of variable amino acids											
	10	**21**	**28**	**70**	**90**	**93**	**123**	**190**	**196**	**207**	**214**	**231**
1	S	N	K	P	V	Q	T	G	T	V	L	F
3	.	.	.	.	.	.	M	.	.	.	.	.
4	.	.	E	.	G	.	.	A	.	L	.	.
5	G	I	.	L	G	H	.	V	.	L	V	L
2	.	S	.	L	G	H	.	V	-	L	V	.

Appendix 4 - GenBank accession numbers for the *GRA6* and *GRA7* loci of the sequenced strains

Strain	GenBank accession numbers	
	GRA6	**GRA7**
RH	AF239283	DQ459443
TgCkCo24	EF512218	EU157142
TgCkBr5	EF512260	EU157172
TgCkBr9	EF512261	EU157173
TgCkBr23	EF512262	EU157174
CCH-2004-NIA	EF512236	EU157175
PSP-2003-KOM	EF512237	EU157176
RMS-2003-DJO	EF512238	EF639859
WIK	EF512239	EU157177
GUY-2003-MEL	EF512241	EU157179
BEVERLEY	AF239284	EU157141
GANGI	EF512219	EU157146
TgCkBr10	EF512243	EU157143
TgCkBr11	EF512244	EU157144
TgCkBr25	EF512245	EU157145
TgCkBr4	EF512246	EU157147
RMS-1994-LEF	EF512220	DQ459452
TgCkAr23	EF512265	EU157155
IPP-2002-URB	EF512224	EU157156
TgCkBr2	EF512264	EU157182
TgCkBr1	EF512251	EU157159

Strain	GenBank accession numbers	
	GRA6	GRA7
TgCkBr18	EF512252	EU157160
CCH-2005-REN	EF512227	EU157161
IPP-2002-BAT	EF512228	—
P80	EF512229	—
TgCkBr16	EF512263	EU157180
MAR-2000-HOU	EF512235	DQ459453
TgCkBr24	EF512253	EU157165
TgCkBr17	EF512256	EU157168
TgCkBr15	EF512257	EU157169
TgCkBr14	EF512258	EU157170
TgCkBr12	EF512259	EU157171
TgCkBr21	EF512254	EU157166
TgCkBr19	EF512255	EU157167
NED	AF239286	DQ459455
ENVL-2002-MAC	EF512222	EU157149
CRL-2004-MOT	EF512221	EU157148
IPB-2003-COG	EF512223	EU157150
TgCkBr3	EF512247	EU157151
TgCkBr22	EF512248	EU157152
TgCkBr6	EF512249	EU157153
TgCkBr7	EF512250	EU157154
TOU-2002-ALI	EF512242	EU157181
GUY-2004-TER	EF512226	EU157158
GUY-2003-BAS	EF512225	EU157157

Strain	GenBank accession numbers	
	GRA6	GRA7
GUY-2004-LAB	EF512230	DQ473315
GUY-2002-MAT	EF512231	EU157162
GUY-2001-DOS	EF512232	DQ459451
GUY-2002-KOE	EF512233	EU157163
GUY-2004-AKO	EF512234	EU157164
P89	EF512240	EU157178
GUY-2004-JAG	DQ187387	EU157183

Appendix 5 – Serotyping results

Table1: Serological reactivity of sera from experimentally infected mice

Strain	6II*	6I/III*	7I*	7III*	Am6*	Am7*	Af6*	Strain type°	GRA6 C-terminal[#]	GRA7 C-terminal[‡]	GRA7 (170–182)[ϕ]	Infection time
RH 1	0.152	0.249	0.058	0.018	–0.022	0.039	0.097	I	GGYGGRADRRPERVY	LQEG	RDTGG	4 weeks
RH 2	0.068	0.207	0.012	–0.008	–0.029	–0.006	–0.017	I	GGYGGRADRRPERVY	LQEG	RDTGG	4 weeks
RH 3	0.171	0.211	0.134	0.024	–0.102	0.046	0.055	I	GGYGGRADRRPERVY	LQEG	RDTGG	4 weeks
RH 1	0.159	0.162	0.045	0.012	–0.035	0.012	0.023	I	GGYGGRADRRPERVY	LQEG	RDTGG	8 weeks
RH 2	0.185	0.162	0.061	0.000	–0.078	0.006	0.023	I	GGYGGRADRRPERVY	LQEG	RDTGG	8 weeks
PT–2005–SUSSCR16–1	0.170	0.355	0.002	–0.022	1.073	–0.017	2.049	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks
PT–2005–SUSSCR16–2	0.178	0.182	0.003	–0.005	0.084	0.001	1.960	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks
PT–2005–SUSSCR16–3	0.224	**0.598**	0.015	–0.006	**0.587**	0.005	**1.276**	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks
PT–2005–SUSSCR16–1	0.216	**0.554**	0.038	**0.228**	**0.853**	0.010	**2.716**	III	RGYGGRGDRRAERVY	PHER	TDSGS	8 weeks
PT–2005–SUSSCR16–2	0.347	**2.073**	0.038	**0.152**	**1.547**	0.097	**2.468**	III	RGYGGRGDRRAERVY	PHER	TDSGS	8 weeks
PT–2005–SUSSCR05–1	0.090	**1.332**	**0.271**	**0.206**	**1.215**	**0.166**	**2.160**	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks
PT–2005–SUSSCR05–2	0.293	**1.844**	—	—	—	—	—	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks

Strain	6II*	6I/III*	7I*	7III*	Am6*	Am7*	Af6*	Strain type°	GRA6 C-terminal[#]	GRA7 C-terminal[∴]	GRA7 (170–182)[ϕ]	Infection time
PT–2005–SUSSCR05–3	0.346	**2.578**	—	—	—	—	—	III	RGYGGRGDRRAERVY	PHER	TDSGS	4 weeks
PT–2005–SUSSCR02	0.078	**1.713**	0.000	**1.346**	**1.292**	–0.092	**2.327**	III	RGYGGRGDRRAERVY	PHER	TDSGS	6 months
LGE–2006–GalDom03–1	**0.679**	0.195	0.038	0.013	0.099	0.008	**0.195**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom03–2	**0.810**	0.178	0.206	**0.116**	**0.378**	**0.105**	**0.186**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom04	**1.879**	0.243	0.142	–0.007	**0.282**	0.037	**0.337**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom05	**1.325**	0.193	0.111	0.015	**1.130**	0.035	**0.163**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom06	**2.641**	0.083	0.069	0.035	**1.325**	0.046	**0.302**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom07	**1.055**	0.070	0.120	0.032	**0.636**	0.057	**0.672**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom08	**1.868**	0.214	0.075	0.051	**1.001**	0.039	**1.770**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom09	**2.251**	0.088	0.041	–0.032	**0.219**	0.010	**0.725**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom10	**2.402**	0.054	0.094	0.024	0.069	0.082	**0.203**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom11	**1.067**	0.098	0.100	0.013	**0.600**	0.066	**0.445**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom12	**1.433**	0.066	0.066	–0.009	**0.115**	0.013	**0.128**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–GalDom13	**1.252**	0.090	0.057	0.011	**0.288**	0.045	**0.440**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks

Strain	6II*	6I/III*	7I*	7III*	Am6*	Am7*	Af6*	Strain type°	GRA6 C-terminal[#]	GRA7 C-terminal[∴]	GRA7 (170–182)[ϕ]	Infection time
LGE–2006–GalDom14	**2.283**	0.089	0.099	0.054	0.049	0.065	**0.282**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
LGE–2006–MEN	**0.410**	0.134	0.055	–0.009	**0.168**	**0.113**	0.033	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
P15–1	**0.439**	0.088	0.173	0.074	**1.031**	0.042	**1.891**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
P15–2	0.108	0.110	0.094	0.068	**0.310**	0.010	0.029	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
P15–3	0.212	0.193	0.167	-0.024	**1.098**	0.078	**0.269**	II	G------DRRPGSEF	LQKG	TETGS	4 weeks
P15	0.172	0.187	—	—	—	—	—	II	G------DRRPGSEF	LQKG	TETGS	9 months
LGE–2006–BRO 1	0.271	0.228	0.087	0.075	**0.749**	**0.116**	**1.395**	II	G------DRRPGSEF	LQKG	TETGS	70 days
LGE–2006–BRO 2	0.214	0.277	**0.375**	0.044	0.047	**0.200**	**0.281**	II	G------DRRPGSEF	LQKG	TETGS	70 days
TOU–2005–HAU1	**1.048**	0.047	0.011	–0.018	**0.166**	–0.026	**0.256**	II	G------DRRPGSEF	LQKG	TETGS	45 days
TOU–2005–HAU3	**0.949**	0.247	0.059	0.020	0.007	0.016	0.109	II	G------DRRPGSEF	LQKG	TETGS	45 days
TOU–2005–CAZ1	**1.197**	**0.918**	0.117	**0.229**	**1.182**	0.038	**2.006**	II	G------DRRPGSEF	LQKG	TETGS	45 days
TOU–2005–CAZ2	**0.845**	0.167	0.086	0.012	**0.803**	0.030	0.099	II	G------DRRPGSEF	LQKG	TETGS	45 days
TOU–2005–CHE1	0.199	0.188	0.014	–0.042	0.106	–0.033	0.027	II	G------DRRPGSEF	LQKG	TETGS	45 days
TOU–2005–CHE2	0.177	0.235	0.013	0.016	–0.016	0.016	0.011	II	G------DRRPGSEF	LQKG	TETGS	45 days

Strain	6II*	6I/III*	7I*	7III*	Am6*	Am7*	Af6*	Strain type°	GRA6 C-terminal[#]	GRA7 C-terminal[∴]	GRA7 (170–182)[ϕ]	Infection time
TOU–2005–CHE3	0.327	0.225	–0.046	–0.096	–0.021	–0.077	0.001	II	G------DRRPGSEF	LQKG	TETGS	45 days
MAR–2000–HOU1	0.142	0.234	—	—	—	—	—	III/II/I	GGYRGRGDRRAEHVY	PHER	TDSGS	4 weeks
MAR–2000–HOU2	0.084	0.171	–0.554	–0.591	–0.593	–0.561	–0.560	III/II/I	GGYRGRGDRRAEHVY	PHER	TDSGS	4 weeks
MAR–2000–HOU3	0.129	0.182	–0.310	–0.291	–0.057	–0.292	–0.192	III/II/I	GGYRGRGDRRAEHVY	PHER	TDSGS	4 weeks
MAR–2000–HOU1	0.128	0.170	0.009	–0.010	–0.011	0.000	0.003	III/II/I	GGYRGRGDRRAEHVY	PHER	TDSGS	8 weeks
MAR–2000–HOU3	0.187	0.288	0.097	–0.084	–0.052	0.035	**0.242**	III/II/I	GGYRGRGDRRAEHVY	PHER	TDSGS	8 weeks
GUY–2004–AKO1	0.151	**0.427**	0.077	–0.061	**0.324**	0.010	**1.505**	atypical	GGYRGRGDRRAEHVY	LQEG	AETSN	4 weeks
GUY–2004–AKO2	0.137	0.197	–0.312	–0.278	**0.301**	–0.284	**1.252**	Atypical	GGYRGRGDRRAEHVY	LQEG	AETSN	4 weeks
GUY–2004–AKO1	0.284	**0.498**	–0.030	**0.138**	**1.231**	0.070	**2.274**	atypical	GGYRGRGDRRAEHVY	LQEG	AETSN	8 weeks
GUY–2004–AKO2	0.151	**0.864**	0.038	–0.009	**0.952**	0.016	**1.617**	Atypical	GGYRGRGDRRAEHVY	LQEG	AETSN	8 weeks
TgCkAr23–1	0.116	0.135	–0.106	–0.154	–0.211	0.004	–0.134	atypical	GGYRGRGDRRAGSVY	PHER	TDSGS	4 weeks
TgCkAr23–2	0.108	0.119	—	—	—	—	—	Atypical	GGYRGRGDRRAGSVY	PHER	TDSGS	4 weeks
TgCkAr23–3	0.047	0.129	0.026	–0.008	–0.017	0.012	0.014	atypical	GGYRGRGDRRAGSVY	PHER	TDSGS	4 weeks
TgCkAr23–1	0.100	0.172	0.008	–0.005	–0.020	–0.008	–0.004	Atypical	GGYRGRGDRRAGSVY	PHER	TDSGS	8 weeks

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value: GRA6 II = 0.369; GRA6 I/III = 0.327; GRA7 I = 0.213; GRA7 III = 0.113; Am6 = 0.109; Am7 = 0.105; Af6 = 0.128. Positive values on bold.

°Genotype defined by the study of five microsatelites markers (*BTUB, TgM A, W35, B17, B18*).

[#]aa polymorphisms from the C-terminal region of GRA6, between position 198 and 230. Residues in red (positions 223, 224, 227, 230) correspond to the region from which peptides GRA6II and GRA6I/III were selected.

∴ aa polymorphisms from the C-terminal region of GRA7 (positions 220, 222, 229, 231).

ϕ aa polymorphisms from the GRA7 region between positions 170 and 182.

Table 2: Serological reactivity against IgG isotypes of sera from experimentally infected mice.

Strain	6II*					6I/III*					Strain type	GRA6 Peptide[#]	Infection time
	IgG1	IgG2a	IgG2b	IgG3	Ig total	IgG1	IgG2a	IgG2b	IgG3	Ig total			
RH 1	0.410	0.345	0.211	0.402	0.152	0.262	0.157	0.077	0.207	0.249	I	ERVY	34 days
RH 2	0.358	0.283	0.233	0.329	0.068	0.069	0.292	0.301	0.285	0.207	I	ERVY	34 days
RH 3	0.392	0.289	0.240	0.379	0.171	0.069	0.264	0.309	0.332	0.211	I	ERVY	34 days
RH 1	0.352	0.349	0.223	0.303	0.159	0.277	0.278	0.305	0.285	0.162	I	ERVY	63 days
RH 2	0.374	**0.394**	0.207	0.389	0.185	0.110	0.130	0.106	0.290	0.162	I	ERVY	63 days
PT–2005–SUSSCR16–1	0361	0.328	0.216	0.462	0.170	0.069	0.341	0.173	0.254	**0.355**	III	ERVY	28 days
PT–2005–SUSSCR16–2	0.326	0.343	0.203	0.373	0.178	0.191	0.184	0.222	0.159	0.182	III	ERVY	28 days
PT–2005–SUSSCR16–3	0.315	0.233	0.104	0.117	0.224	0.235	**1.590**	0.163	0.158	**0.598**	III	ERVY	28 days
PT–2005–SUSSCR16–1	0.374	0.337	0.192	0.357	0.216	0.184	**1.271**	0.294	0.382	**0.554**	III	ERVY	56 days
PT–2005–SUSSCR16–2	0.407	**0.414**	0.216	0.379	0.347	0.383	**2.589**	2.247	1.907	**2.073**	III	ERVY	56 days
PT–2005–SUSSCR05–1	0.158	0.180	0.138	0.119	0.090	0.164	**2.447**	0.286	0.695	**1.332**	III	ERVY	28 days
PT–2005–SUSSCR05–2	0.320	0.208	0.123	0.132	0.293	0.127	**0.503**	0.165	0.147	**1,844**	III	ERVY	28 days

Strain	6II*					6I/III*					Strain type	GRA6 Peptide[#]	Infection time
	IgG1	IgG2a	IgG2b	IgG3	Ig total	IgG1	IgG2a	IgG2b	IgG3	Ig total			
PT–2005–SUSSCR05–3	0.179	—	—	—	0.346	0.126	**2.061**	0.212	0.139	**2.578**	III	ERVY	28 days
PT–2005–SUSSCR02	0.056	0.073	0.064	0.062	0.078	0.071	**1.541**	0.111	0.107	**1.713**	III	ERVY	6 months
LGE–2006–GalDom03–1	0.325	**0.564**	0.202	0.392	**0.679**	0.168	0.360	0.277	0.291	0.195	II	GSEF	1 month
LGE–2006–GalDom03–2	0.175	**0.447**	0.120	0.232	**0.810**	0.092	0.217	0.116	0.114	0.178	II	GSEF	1 month
LGE–2006–GalDom04	0.101	**1.002**	0.120	0.056	**1.879**	0.102	0.304	0.137	0.133	0.243	II	GSEF	1 month
LGE–2006–GalDom05	0.114	**3.081**	0.157	0.199	**1.325**	0.148	0.167	0.116	0.109	0.193	II	GSEF	1 month
LGE–2006–GalDom06	0.158	**2.087**	0.264	0.084	**2.641**	0.081	0.164	0.096	0.095	0.083	II	GSEF	1 month
LGE–2006–GalDom07	0.118	**2.824**	0.225	0.129	**1.055**	0.140	0.244	0.107	0.119	0.070	II	GSEF	1 month
LGE–2006–GalDom08	0.106	**3.415**	0.087	0.082	**1.868**	0.074	0.201	0.072	0.077	0.214	II	GSEF	1 month
LGE–2006–GalDom09	0.143	**0.557**	0.143	0.108	**2.251**	0.183	0.335	0.259	0.172	0.088	II	GSEF	1 month
LGE–2006–GalDom10	0.141	**1.197**	0.269	0.187	**2.402**	0.152	0.189	0.099	0.113	0.054	II	GSEF	1 month
LGE–2006–GalDom11	0.134	**0.504**	0.217	0.118	**1.067**	0.116	0.317	0.312	0.271	0.098	II	GSEF	1 month

Strain	6II*					6I/III*					Strain type	GRA6 Peptide[#]	Infection time
	IgG1	IgG2a	IgG2b	IgG3	Ig total	IgG1	IgG2a	IgG2b	IgG3	Ig total			
LGE–2006–GalDom12	0.157	**1.844**	0.219	0.197	**1.433**	0.111	0.113	0.297	0.202	0.066	II	GSEF	1 month
LGE–2006–GalDom13	0.133	0.169	0.097	0.123	**1.252**	0.108	0.121	0.242	0.132	0.090	II	GSEF	1 month
LGE–2006–GalDom14	0.184	0.341	0.132	0.162	**2.283**	0.097	0.283	0.247	0.186	0.089	II	GSEF	1 month
LGE–2006–MEN	0.206	0.345	0.126	0.169	**0.410**	0.089	0.295	0.272	0.116	0.134	II	GSEF	1 month
P15–1	0.188	0.188	0.119	0.209	**0.439**	0.153	0.354	0.288	0.161	0.088	II	GSEF	28 days
P15–2	0.171	0.102	0.126	0.166	0.108	0.156	0.272	0.275	0.142	0.110	II	GSEF	28 days
P15–3	0.174	0.309	0.180	0.169	0.212	0.163	0.183	0.318	0.164	0.193	II	GSEF	28 days
P15	0.256	0.136	0.087	0.146	0.172	0.089	0.167	0.125	0.102	0.187	II	GSEF	9 months
LGE–2006–BRO1	0.169	**0.492**	0.108	0.113	0.271	0.202	0.294	0.251	0.097	0.228	II	GSEF	70 days
LGE–2006–BRO2	0.142	**0.686**	0.208	0.164	0.214	0.223	0.238	0.185	0.106	0.277	II	GSEF	70 days
TOU–2005–HAU1	0.350	**1.121**	0.330	0.148	**1.048**	0.158	0.128	0.096	0.116	0.047	II	GSEF	45 days
TOU–2005–HAU3	0.265	0.252	0.141	0.159	**0.949**	0.208	0.340	0.330	0.178	0.247	II	GSEF	45 days

Strain	6II*					6I/III*					Strain type	GRA6 Peptide[#]	Infection time
	IgG1	IgG2a	IgG2b	IgG3	Ig total	IgG1	IgG2a	IgG2b	IgG3	Ig total			
TOU–2005–CAZ 1	0.296	**0.555**	0.212	0.175	**1.197**	0.204	**0.680**	0.300	0.115	**0.918**	II	GSEF	45 days
TOU–2005–CAZ 2	0.190	**0.532**	0.222	0.214	**0.845**	0.179	0.431	0.267	0.179	0.167	II	GSEF	45 days
TOU–2005–CHE 1	0.144	0.100	0.089	0.097	0.199	0.113	0.299	0.149	0.093	0.188	II	GSEF	45 days
TOU–2005–CHE 2	0.120	0.140	0.134	0.074	0.177	0.216	0.127	0.281	0.117	0.235	II	GSEF	45 days
TOU–2005–CHE 3	0.278	0.246	0.217	0.089	0.327	0.208	0.150	0.166	0.131	0.225	II	GSEF	45 days
MAR–2000–HOU1	0.199	0.230	0.085	0.264	0.142	0.096	0.068	0.095	0.106	0.234	III/II/I	EHVY	28 days
MAR–2000–HOU2	0.264	**0.369**	0.083	0.074	0.084	0.127	0.092	0.082	0.116	0.171	III/II/I	EHVY	28 days
MAR–2000–HOU3	0.144	0.192	0.098	0.087	0.129	0.066	0.081	0.097	0.065	0.182	III/II/I	EHVY	28 days
MAR–2000–HOU1	0.150	0.193	0.235	0.141	0.128	0.085	0.081	0.092	0.096	0.170	III/II/I	EHVY	56 days
MAR–2000–HOU3	0.223	0.311	0.109	0.161	0.187	0.122	0.120	0.088	0.078	0,288	III/II/I	EHVY	56 days
GUY–2004–AKO1	0.305	0.223	0.252	0.149	0.151	0.540	0.246	0.206	0.080	**0.427**	Atypical	EHVY	28 days
GUY–2004–AKO2	0.306	0.265	0.133	0.099	0.137	0.484	0.112	0.255	0.246	0.197	Atypical	EHVY	28 days

Strain	6II*					6I/III*					Strain type	GRA6 Peptide[#]	Infection time
	IgG1	IgG2a	IgG2b	IgG3	Ig total	IgG1	IgG2a	IgG2b	IgG3	Ig total			
GUY–2004–AKO1	0.372	**0.571**	0.131	0.152	0.284	0.106	**0.617**	0.309	0.279	**0.498**	Atypical	EHVY	56 days
GUY–2004–AKO2	0.145	0.248	0.102	0.249	0.151	0.138	**1.679**	0.230	0.114	**0.864**	Atypical	EHVY	56 days
TgCkAr23–1	0.170	0.174	0.075	0.107	0.116	0.075	0.059	0.084	0.090	0.135	Atypical	GSVY	28 days
TgCkAr23–2	0.235	0.258	0.208	0.217	0.108	0.109	0.093	0.266	0.136	0.119	Atypical	GSVY	28 days
TgCkAr23–3	0.353	0.202	0.092	0.129	0.047	0.147	0.077	0.097	0.068	0.129	atypical	GSVY	28 days
TgCkAr23–1	0.287	0.209	0.094	0.099	0.100	0.131	0.159	0.135	0.135	0.172	Atypical	GSVY	56 days

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value for IgG: GRA6 II = 0.369; GRA6 I/III = 0.327. Cut off value for IgG2a: GRA6 II = 0.354; GRA6 I/III = 0.496. Positive values on bold.

° Genotype defined by the study of five microsatelites markers (*BTUB, TgM A, W35, B17, B18*).

[#] aa polymorphisms from the C-terminal region of GRA6 (positions 223, 224, 227, 230).

Table 3: Serological reactivity of human infections with GRA6 and GRA7 specific peptides for archetypal strains.

Strain	Pathology	Origin	6II*	6I/III*	7I*	7III*	GRA6 C-terminal[#]	GRA7 C-terminal[#]	Genotype°
A^+	Congenital (M)	France	0.063	-0.003	-0.034	-0.019	GSEF	LQKG	II
A^+	Congenital (C)	France	0.054	-0.029	0.019	0.016	GSEF	LQKG	II
B^+	Congenital (M)	France	0.082	-0.052	0.022	0.058	GSEF	LQKG	II
B^+	Congenital (C)	France	**0.269**	-0.019	0.002	0.012	GSEF	LQKG	II
LGE-01-4	Congenital (M)	France	**1.471**	**0.792**	0.017	0.012	GSEF	LQKG	II
LGE-01-4	Congenital (C)	France	**1.666**	**1.930**	0.072	0.073	GSEF	LQKG	II
LGE-01-1	Congenital (M)	France	0.005	-0.041	**0.123**	0.083	GSEF	LQKG	II
LGE-01-1	Congenital (C)	France	**0.302**	-0.025	0.080	0.041	GSEF	LQKG	II
LGE-01-2	Congenital (M)	France	0.060	0.003	0.022	0.007	GSEF	LQKG	II
LGE-01-2	Congenital (C)	France	0.071	0.023	0.012	0.058	GSEF	LQKG	II
LGE-00-2	Congenital (M)	France	**0.096**	-0.011	0.050	0.012	GSEF	LQKG	II
LGE-00-2	Congenital (C)	France	0.084	-0.005	0.096	0.048	GSEF	LQKG	II
LGE-2003-DUP	Congenital (M)	France	0.054	-0.008	0.033	0.091	GSEF	LQKG	II
LGE-2003-DUP	Congenital (C)	France	**0.432**	-0.044	0.026	0.108	GSEF	LQKG	II

Strain	Pathology	Origin	6II*	6I/III*	7I*	7III*	GRA6 C-terminal[#]	GRA7 C-terminal[#]	Genotype°
LGE-2004-ALB	Congenital (M)	France	**0.330**	0.023	0.032	0.016	GSEF	LQKG	II
LGE-2003-BAS	Congenital (M)	France	0.046	-0.018	0.059	0.080	GSEF	LQKG	II
LGE-2003-BAS	Congenital (C)	France	**2.088**	-0.004	-0.003	-0.002	GSEF	LQKG	II
LGE-2002-FAY	Congenital (M)	France	**0.217**	-0.018	0.018	0.008	GSEF	LQKG	II
LGE-2006-MOR	Congenital (M)	France	0.049	-0.015	0.024	0.107	GSEF	LQKG	II
LGE-2006-MOR	Congenital (C)	France	**0.110**	0.002	0.084	0.074	GSEF	LQKG	II
LGE-00-1	Congenital (M)	France	**0.743**	0.007	0.021	0.029	GSEF	LQKG	II
LGE-00-1	Congenital (C)	France	**0.298**	-0.032	0.060	0.046	GSEF	LQKG	II
LGE-2006-MEN	Congenital (M)	France	0.082	0.003	0.046	0.114	GSEF	LQKG	II
LGE-2006-MEN	Congenital (C)	France	**0.279**	-0.029	0.007	0.013	GSEF	LQKG	II
LGE-2005-FRA	Congenital (M)	France	**0.107**	-0.015	**0.123**	**0.215**	GSEF	LQKG	II
LGE-2005-FRA	Congenital (C)	France	**0.195**	-0.025	0.028	0.069	GSEF	LQKG	II
LGE-2004-VIL	Congenital (M)	France	**0.354**	-0.019	0.015	0.026	GSEF	LQKG	II

Strain	Pathology	Origin	6II*	6I/III*	7I*	7III*	GRA6 C-terminal[#]	GRA7 C-terminal[#]	Genotype°
LGE-2004-VIL	Congenital (C)	France	**0.183**	-0.034	0.055	0.043	GSEF	LQKG	II
LGE-2004-CAI	Congenital (M)	France	**0.307**	-0.025	0.025	0.050	GSEF	LQKG	II
LGE-2004-CAI	Congenital (C)	France	**0.150**	-0.007	0.021	0.010	GSEF	LQKG	II
LGE-2005-COL	Congenital (M)	France	**0.225**	-0.040	0.010	0.023	GSEF	LQKG	II
LGE-2005-COL	Congenital (C)	France	**0.218**	0.000	0.097	0.052	GSEF	LQKG	II
LGE-2007-LOR	Congenital (M)	France	**0.122**	-0.03	0.073	0.044	GSEF	LQKG	II
LGE-2007-LOR	Congenital (C)	France	**0.159**	-0.007	0.057	0.038	GSEF	LQKG	II
NED	Congenital (M)	France	0.020	**0.202**	0.024	0.064	ERVY	PHER	III
NED	Congenital (C)	France	-0.031	**0.123**	0.047	**1.137**	ERVY	PHER	III
LGE-97-3	Congenital (M)	France	0.053	**0.131**	0.012	**0.294**	ERVY	PHER	III
LGE-97-3	Congenital (C)	France	0.011	**0.118**	**0.152**	**0.820**	ERVY	PHER	III
TOU-2004-FEU	Congenital (M)	France	0.032	**0.385**	0.018	0.028	ERVY	PHER	III
TOU-2004-FEU	Congenital (C)	France	0.008	**0.312**	0.019	0.037	ERVY	PHER	III
RH	Lymphadenopathy	France	0.019	**0.174**	0.026	0.035	ERVY	LQEG	I

Strain	Pathology	Origin	6II*	6I/III*	7I*	7III*	GRA6 C-terminal[#]	GRA7 C-terminal[#]	Genotype°
RH	Lymphadenopathy	France	0.081	**1.149**	0.040	0.046	ERVY	LQEG	I
RH	Lymphadenopathy	France	0.058	**1.115**	0.043	0.053	ERVY	LQEG	I
RH	Lymphadenopathy	France	0.007	**0.133**	0.042	0.056	ERVY	LQEG	I
RH	Lymphadenopathy	Portugal	-0.057	**0.184**	0.031	0.007	ERVY	LQEG	I
RH	Lymphadenopathy	Portugal	0.006	**0.230**	0.005	0.055	ERVY	LQEG	I
RMS-2006-PAR	Asymptomatic	France	**0.312**	0.002	0.030	0.034	GSEF	LQKG	II
GUY-2004-TER	Congenital (M)	Suriname	**0.372**	**0.471**	0.049	0.092	GSVY	LQEG	Atypical
GUY-2004-TER	Congenital (M)	Suriname	**0.371**	**0.588**	0.045	0.069	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.019	**0.333**	0.058	0.094	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	**0.484**	-0.022	0.017	0.068	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.052	-0.009	-0.053	-0.031	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.034	0.045	0.113	0.018	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	**0.139**	**0.586**	0.004	0.017	GSVY	LQEG	Atypical
GUY-2004-TER	Multivisceral	Suriname	-0.073	**0.144**	0.067	-0.002	GSVY	LQEG	Atypical

Strain	Pathology	Origin	6II*	6I/III*	7I*	7III*	GRA6 C-terminal[#]	GRA7 C-terminal[#]	Genotype°
GUY-2004-TER	Multivisceral	Suriname	**0.148**	**0.145**	0.061	**0.331**	GSVY	LQEG	Atypical
GUY-2004-TER	Asymptomatic	Suriname	0.019	0.009	0.053	0.073	GSVY	LQEG	Atypical
GUY-2004-TER	Asymptomatic	Suriname	**0.943**	**2.346**	**0.781**	**1.688**	GSVY	LQEG	Atypical
GUY-2004-TER	Asymptomatic	Suriname	0.042	0.024	0.047	0.004	GSVY	LQEG	Atypical
GUY-2003-BAS	Multivisceral	French Guiana	**0.121**	0.030	0.094	0.079	GSVY	LQEG	Atypical
GUY-2004-AKO	Multivisceral	French Guiana	0.054	**0.838**	0.044	0.096	EHVY	LQEG	Atypical
GUY-2002-KOE	Multivisceral	French Guiana	0.022	**0.476**	0.027	**0.733**	ERVY	LQEG	Atypical
GUY-2002-MAT	Multivisceral	French Guiana	0.076	**1.241**	0.062	0.080	ERVY	LQEG	Atypical

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value for European samples: GRA6 II = 0.088; GRA6 I/III = 0.076; GRA7 I = 0.114; GRA7 III = 0.126. Cut off value for South American samples: GRA6 II = 0.089; GRA6 I/III = 0.067; GRA7 I = 0.124; GRA7 III = 0.120. Positive values on bold.

[+] Strain not isolated. A and B identifies the serum samples associated with the two congenital infections.

[#] aa polymorphisms from the C-terminal region of GRA6 (positions 223, 224, 227, 230) and GRA7 (positions 220, 222, 229, 231).

° Genotype defined by the study of five microsatelites markers (*BTUB, TgM A, W35, B17, B18*).

(M), indicates serum samples from mother and (C), indicates serum samples from child.

Table 4: Serological reactivity of human infections with GRA6 and GRA7 specific peptides for non-archetypal strains.

Strain	Pathology	Origin	Am6*	Am7*	Af6*	GRA6 aa[#]	GRA7 aa[#]	Genotype°
LGE-01-1	Congenital (C)	France	0.041	0.015	-0.003	G------DRRPGSEF	TETGS	II
LGE-00-2	Congenital (M)	France	**0.193**	0.038	**0.153**	G------DRRPGSEF	TETGS	II
LGE-2003-DUP	Congenital (C)	France	**0.998**	0.101	**0.202**	G------DRRPGSEF	TETGS	II
LGE-2003-BAS	Congenital (C)	France	**0.268**	0.086	0.086	G------DRRPGSEF	TETGS	II
LGE-00-1	Congenital (M)	France	**0.586**	0.072	**0.174**	G------DRRPGSEF	TETGS	II
LGE-2005-FRA	Congenital (M)	France	0.096	-0.011	0.008	G------DRRPGSEF	TETGS	II
LGE-2004-CAI	Congenital (M)	France	**0.293**	0.022	**0.319**	G------DRRPGSEF	TETGS	II
LGE-2007-LOR	Congenital (M)	France	**0.339**	0.122	**0.147**	G------DRRPGSEF	TETGS	II
TOU-2004-FEU	Congenital (M)	France	**0.541**	0.056	**0.727**	RGYGGRGDRRAERVY	TDSGS	III
RH	Lymphadenopathy	France	**0.987**	0.108	**1.197**	GGYGGRADRRPERVY	RDTGG	I
GUY-2004-TER	Congenital (M)	Suriname	**0.533**	0.026	**0.404**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Congenital (M)	Suriname	**1.223**	0.114	**0.555**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	**0.749**	-0.088	**0.488**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.215	-0.075	-0.081	GGYRGRGDRRAGSVY	AETSN	Atypical

Strain	Pathology	Origin	Am6*	Am7*	Af6*	GRA6 aa#	GRA7 aa#	Genotype°
GUY-2004-TER	Multivisceral	Suriname	0.139	**0.428**	**0.956**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.015	0.032	-0.001	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	0.082	-0.008	**0.188**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	**0.524**	**0.852**	**0.577**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Multivisceral	Suriname	-0.01	0.255	**0.142**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Asymptomatic	Suriname	-0.027	-0.029	-0.042	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Asymptomatic	Suriname	**1.411**	0.254	**1.755**	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-TER	Asymptomatic	Suriname	0.028	0.038	0.066	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2003-BAS	Multivisceral	French Guiana	0.024	0.058	0.028	GGYRGRGDRRAGSVY	AETSN	Atypical
GUY-2004-AKO	Multivisceral	French Guiana	**0.513**	0.096	**0.656**	GGYRGRGDRRAEHVY	AETSN	Atypical
GUY-2002-KOE	Multivisceral	French Guiana	**0.868**	**0.372**	**1.109**	GGYRGRGDRRAERVY	AETSN	Atypical
GUY-2002-MAT	Multivisceral	French Guiana	**2.362**	0.11	**1.703**	GGYRGRGDRRAERVY	AETSN	Atypical

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value for European samples: Am6 = 0.180; Am7 = 0.188; Af6 = 0.096. Cut off value for South American samples: Am6 = 0.252; Am7 = 0.270; Af6 = 0.079. Positive values on bold.

[#] aa polymorphisms from the C-terminal region of GRA6 (positions 198, 204-208, 213, 216-219, 223, 224, 227, 230) and GRA7 (positions 170, 172, 176, 178, 182).

° Genotype defined by the study of five microsatelites markers (*BTUB, TgM A, W35, B17, B18*).

(M), indicates serum samples from mother and (C), indicates serum samples from child.

Table 5: Serological reactivity of chicken sera with GRA6 and GRA7 specific peptides.

Serum	Strain	Geographical origin	GRA6II*	GRA6I/III*	GRA7I*	GRA7III*	Am6*	Am7*	Af6*	Genotype°
GA40	TgCkPr4	Portugal	0.025	0.018	0.053	0.092	0.027	0.067	0.068	II
GA147	TgCkPr6	Portugal	**0.104**	0.004	0.012	0.051	0.087	0.035	0.035	II
GA163	TgCkPr8	Portugal	**0.990**	0.014	0.113	0.181	**0.610**	0.111	**0.393**	II
GA164	TgCkPr9	Portugal	**0.170**	0.003	**0.279**	0.230	**0.635**	0.099	0.077	II
GA166	TgCkPr7	Portugal	**0.316**	-0.005	0.070	0.073	**0.289**	0.109	**0.350**	II
GA167	TgCkPr10	Portugal	**0.319**	**0.221**	0.081	0.116	**0.234**	0.108	0.288	II
GA170	TgCkPr11	Portugal	0.026	0.008	0.069	0.097	**0.119**	0.107	0.116	II
GA176	TgCkPr12	Portugal	0.053	-0.013	0.041	0.086	0.019	0.056	0.070	II
GA19	TgCkPr1	Portugal	0.023	-0.009	0.123	0.065	0.077	**0.221**	0.195	III
GA39	TgCkPr3	Portugal	0.040	0.005	0.049	0.043	**0.181**	0.077	0.077	III
GA43	TgCkPr5	Portugal	0.038	**0.902**	0.089	0.169	**0.517**	0.079	**0.852**	III

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value: GRA6 II = 0.058; GRA6 I/III = 0.051; GRA7 I = 0.180; GRA7 III = 0.244; Am6 = 0.115; Am7 = 0.195; Af6 = 0.322. Positive values on bold.

°Genotype defined by the study of *SAG2* locus by PCR-RFLP. GA147, GA163, GA164 and GA167 were also genotyped by five MS markers (*BTUB, TgM A, W35, B17, B18*).

Table 6: Serological reactivity of pig sera with GRA6 and GRA7 specific peptides

Serum	Strain	Geographical origin	GRA6II∴	GRA6I/III∴	GRA7I∴	GRA7III∴	Am6∴	Am7∴	Af6∴	Genotype°
PV44	PT2005SUSSCR01	Portugal	0.104	0.052	-0.017	0.162	0.091	0.046	0.160	II
PV214	PT2005SUSSCR04	Portugal	0.097	0.107	-0.023	0.054	0.135	0.021	0.119	II
PV227	PT2005SUSSCR06	Portugal	**0.824**	-0.080	-0.018	0.134	0.199	0.055	0.242	II
PV231	PT2005SUSSCR07	Portugal	**0.967**	**0.276**	-0.090	**0.720**	0.265	0.012	0.255	II
PV232	PT2005SUSSCR10	Portugal	0.185	**0.544**	0.044	0.069	**0.702**	0.164	**1.094**	II
PV266	PT2005SUSSCR09	Portugal	-0.114	-0.125	-0.156	-0.059	0.039	-0.071	0.069	II
PV272	PT2005SUSSCR08	Portugal	**0.742**	**0.477**	0.032	0.033	0.548	0.100	0.566	II
PV274	PT2005SUSSCR13	Portugal	**1.074**	**0.576**	0.020	0.036	**0.922**	0.136	**0.932**	II
PV282	PT2005SUSSCR12	Portugal	0.044	-0.093	0.049	0.035	0.299	0.159	**0.917**	II
PV302	PT2005SUSSCR15	Portugal	-0.080	-0.073	-0.019	0.042	0.531	0.052	**0.712**	II
PV238	PT2005SUSSCR11	Portugal	**0.615**	0.020	-0.024	0.048	0.470	0.276	0.685	II*
PV116	PT2005SUSSCR02	Portugal	0.008	-0.089	0.019	0.186	0.241	0.108	0.355	III
PV220	PT2005SUSSCR05	Portugal	-0.056	-0.099	0.011	0.028	0.094	0.101	0.462	III
PV311	PT2005SUSSCR14	Portugal	-0.023	0.080	0.005	0.020	0.291	0.225	0.307	III
PV316	PT2005SUSSCR16	Portugal	0.017	0.005	0.008	0.033	**0.849**	0.050	0.130	III

∴OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value: GRA6 II = 0.212; GRA6 I/III = 0.249; GRA7 I = 0.231; GRA7 III = 0.228; Am6 = 0.549; Am7 = 0.403; Af6 = 0.704. Positive values on bold.

°Genotype defined by the study of five microsatellite markers (*BTUB, TgM A, W35, B17, B18*).

*Strain with an atypical allele (5) for the *W35* locus.

Table 7: Optical density values for human serum samples from Europe and African countries against the atypical peptides.

Sera	Geographic Origin	Peptides*			Disease
		Am6	Am7	Af6	
F1º	France	-0.410	-0.392	-0.376	acute
F44º	France	0.042	0.017	0.016	ocular
P10º	Portugal	0.016	0.007	-0.005	congenital (mother)
P11º	Portugal	0.088	0.015	0.060	congenital (mother)
P12	Portugal	0.016	0.016	0.003	congenital (mother)
P13º	Portugal	**0.339**	0.047	**0.315**	congenital (mother)
P14º	Portugal	0.091	0	0.095	congenital (mother)
P15	Portugal	0.015	0.029	0.062	congenital (mother)
P16º	Portugal	-0.004	-0.010	0.010	congenital (mother)
P17º	Portugal	0.063	-0.015	-0.019	congenital (mother)
P18º	Portugal	0.106	-0.003	-0.016	congenital (mother)
P19º	Portugal	0.162	0.008	**0.100**	congenital (mother)
P20º	Portugal	0.024	0.027	0.029	congenital (mother)
F32º	France	0.019	0.014	0.019	congenital (mother)
F33º	France	0.096	0.012	0.007	congenital (child)
F34º	France	0.051	-0.026	-0.041	congenital (child)
P21	Portugal	0.032	0.019	**0.098**	HIV
P22º	Portugal	0.105	0.020	**0.166**	HIV
F35º	France	**0.358**	0.020	**0.372**	HIV
F36º	France	**0.483**	0.004	**0.143**	HIV
F37º	France	**0.269**	-0.103	**0.246**	HIV
F38º	France	**0.228**	0.004	**0.190**	HIV

Sera	Geographic Origin	Peptides*			Disease
		Am6	Am7	Af6	
F39	France	0.025	-0.005	0.047	HIV
F40º	France	0.057	0.014	-0.006	HIV
F41º	France	**0.436**	0.028	**0.404**	HIV
F42	France	0.151	0.039	0.085	HIV
F43º	France	**0.239**	0.021	**0.191**	HIV
P1	Portugal	0.070	0.025	0.035	asymptomatic
P2º	Portugal	0.172	0.025	**0.284**	asymptomatic
P3º	Portugal	-0.011	0.010	-0.005	asymptomatic
P4º	Portugal	0.164	0.023	**0.182**	asymptomatic
P5	Portugal	0.010	0.022	0.017	asymptomatic
P6º	Portugal	0.042	0.021	0.020	asymptomatic
P7º	Portugal	0.114	0.019	0	asymptomatic
P8º	Portugal	0.034	0.013	0.023	asymptomatic
P9º	Portugal	**0.194**	-0.003	**0.224**	asymptomatic
F2º	France	-0.008	-0.003	-0.006	asymptomatic
F3	France	-0.008	0	0.004	asymptomatic
F4º	France	0.067	0.015	0.034	asymptomatic
F5º	France	**0.416**	-0.015	**0.294**	asymptomatic
F6	France	-0.003	0.003	0.015	asymptomatic
F7º	France	0.103	0.030	0.075	asymptomatic
F8	France	-0.004	0.020	0.017	asymptomatic
F9º	France	-0.002	-0.003	-0.005	asymptomatic

Sera	Geographic Origin	Peptides*			Disease
		Am6	Am7	Af6	
F10	France	-0.013	0.008	-0.001	asymptomatic
F11	France	0.009	0.008	0.006	asymptomatic
F12	France	0.012	0.016	0.053	asymptomatic
F13	France	-0.006	0.008	-0.008	asymptomatic
F14º	France	0.019	0.001	0.012	asymptomatic
F15º	France	0.014	-0.003	0.012	asymptomatic
F16º	France	**0.433**	0.057	**0.269**	asymptomatic
F17	France	-0.005	0.012	-0.002	asymptomatic
F18º	France	0.144	0.001	0.073	asymptomatic
F19º	France	**0.466**	-0.001	**0.329**	asymptomatic
F20º	France	0.047	0.012	0.075	asymptomatic
F21º	France	-0.017	-0.092	-0.057	asymptomatic
F22	France	0.065	0.025	0.037	asymptomatic
F23	France	-0.016	0.021	-0.001	asymptomatic
F24º	France	**0.255**	0.007	-0.001	asymptomatic
F25º	France	0.056	0	0.066	asymptomatic
F26	France	0.028	-0.001	-0.002	asymptomatic
F27º	France	-0.001	0.012	0.046	asymptomatic
F28	France	0.009	0.010	0.012	asymptomatic
F29	France	-0.009	0.020	0.004	asymptomatic
F30	France	0.009	0.010	-0.012	asymptomatic
F31	France	-0.007	0.049	0.011	asymptomatic

Sera	Geographic Origin	Peptides*			Disease
		Am6	Am7	Af6	
IC1°	Ivory Coast	**0.454**	-0.057	**1.514**	asymptomatic
IC3	Ivory Coast	0.001	0.030	0.119	asymptomatic
IC5	Ivory Coast	0.075	-0.074	0.027	asymptomatic
IC7	Ivory Coast	0.101	0.024	0.131	asymptomatic
IC8°	Ivory Coast	**0.937**	0.065	**0.365**	asymptomatic
IC11	Ivory Coast	**0.139**	0.021	0.104	asymptomatic
IC15	Ivory Coast	**0.133**	**0.731**	**0.569**	asymptomatic
IC16°	Ivory Coast	**0.354**	0.004	**0.645**	asymptomatic
IC17°	Ivory Coast	**0.232**	-0.049	0.09	asymptomatic
IC18	Ivory Coast	0.072	-0.045	0.015	asymptomatic
IC19	Ivory Coast	0.115	0.032	0.214	asymptomatic
IC20	Ivory Coast	0.025	0.038	0.115	asymptomatic
IC21	Ivory Coast	**0.214**	0.019	0.278	asymptomatic
IC22°	Ivory Coast	**0.909**	**0.445**	**1.255**	asymptomatic
IC23°	Ivory Coast	**1.887**	0.020	**2.343**	asymptomatic
IC24°	Ivory Coast	**0.419**	0.053	0.348	asymptomatic
IC26	Ivory Coast	0.024	0.052	0.156	asymptomatic
IC27°	Ivory Coast	**0.153**	0.035	0.047	asymptomatic
IC28	Ivory Coast	**0.154**	**0.304**	0.083	asymptomatic
IC29	Ivory Coast	-0.045	-0.064	-0.103	asymptomatic
IC30°	Ivory Coast	**0.394**	**1.894**	0.272	asymptomatic
IC31°	Ivory Coast	**0.154**	**0.232**	0.170	asymptomatic

Sera	Geographic Origin	Peptides*			Disease
		Am6	Am7	Af6	
IC32	Ivory Coast	0.098	**0.490**	0.098	asymptomatic
IC34	Ivory Coast	**0.562**	**0.197**	**0.565**	asymptomatic
IC35	Ivory Coast	**0.184**	0.121	0.093	asymptomatic
IC36	Ivory Coast	-0.006	0.143	0.052	asymptomatic
IC38	Ivory Coast	**0.153**	**0.218**	0.160	asymptomatic
IC42	Ivory Coast	0.028	0.154	0.148	asymptomatic
IC45	Ivory Coast	0.096	0.081	0.116	asymptomatic
IC47	Ivory Coast	**0.159**	0.157	-0.032	asymptomatic
IC48°	Ivory Coast	0.030	**0.271**	0.004	asymptomatic
IC49	Ivory Coast	-0.044	**0.479**	0.014	asymptomatic
IC51	Ivory Coast	0.048	0.037	-0.027	asymptomatic
IC54°	Ivory Coast	**1.699**	0.161	**1.281**	asymptomatic
IC55°	Ivory Coast	**2.098**	0.057	**1.441**	asymptomatic
IC56	Ivory Coast	**0.531**	0.108	**0.420**	asymptomatic
IC57°	Ivory Coast	**0.143**	**0.302**	0.115	asymptomatic
IC58°	Ivory Coast	**0.527**	**0.552**	**0.413**	asymptomatic

*OD index calculated by subtracting the OD of the peptide control from the OD of each peptide. Cut off value for European countries: Am6 = 0.180; Am7 = 0.188; Af6 = 0.096. Cut off value for Ivory Coast samples: Am6 = 0.122; Am7 = 0.170; Af6 = 0.360. Positive values on bold.

° Serum samples that have also reacted with the peptides specific for the archetypal strains.