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Strain hypothesis of *Toxoplasma gondii* infection on the outcome of human diseases

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Abstract

The intracellular protozoan *Toxoplasma gondii* is an exceptionally successful food- and waterborne parasite that infects approximately 1 billion people worldwide. Genotyping of *T. gondii* isolates from all continents revealed a complex population structure. Recent research supports the notion that *T. gondii* genotype may be associated with disease severity. Here, we (1) discuss molecular and serological approaches for designation of *T. gondii* strain type, (2) overview the literatures on the association of *T. gondii* strain type and the outcome of human disease, and (3) explore possible mechanisms underlying these strain specific pathology and severity of human toxoplasmosis. Although no final conclusions can be drawn, it is clear that virulent strains (e. g. strains containing type I or atypical alleles) are significantly more often associated with increased frequency and severity of human toxoplasmosis. The significance of highly virulent strains can cause severe diseases in immunocompetent patients and might implicated in brain disorders such as schizophrenia should led to reconsideration of toxoplasmosis. Further studies that combine parasite strain typing and human factor analysis (e.g. immune status and genetic background) are required for better understanding of human susceptibility or resistance to toxoplasmosis.

Keywords

Toxoplasma gondii; strain typing; virulence; outcome of human infection; human factors; mechanisms; toxoplasmosis

Introduction

Toxoplasma gondii is a widespread protozoan parasite in the phylum Apicomplexa. This phylum comprises more than 5000 species, only a few of which (e.g. *Plasmodium* spp. and *Cryptosporidium* spp.) cause disease in humans (Dubey 2010). *T. gondii* has been considered as one of the most successful eukaryotic pathogens concerning the number of host species and percentage of animals infected globally. The prevalence of humans infection varies from 15 to 85% worldwide depending on geographical location (Dubey & Beattie, 1988). Most infections are asymptomatic or take the form of a mild, self-limiting

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Conflict of interest

The authors declare that they have no conflicts of interest.

illness characterized by fever, malaise and lymphadenopathy. Nevertheless, in situations of immunodeficiency (i.e., AIDS, and transplant and cancer patients receiving immunosuppressive medication) or when the parasite is congenitally acquired, *T. gondii* can cause severe disease even death if not treated properly.

T. gondii has a complex life cycle involving sexual replication in members of the cat family (Felidae) and asexual propagation in a wide variety of warm-blooded hosts (Dubey, 2008). There are three infectious stages in the life cycle of *T. gondii*: tachyzoites, which facilitate expansion during acute infection, bradyzoites, which maintain chronic infection, and sporozoites, which are disseminated in the environment within oocysts (Sibley *et al.* 2009). While infection elicits strong innate and adaptive immune responses to control parasite multiplication, they do not eradicate the infection. Consequently, the host is left with life-long latent infection characterized by the presence of tissue cysts. Tissue cysts form in a variety of cells, especially long-lived differentiated cells such as neurons and muscle cells, thus assuring long-term infection (Sibley *et al.* 2009). In humans, the tropism of *T. gondii* for brain has been suggested to account for some mental disorders including schizophrenia, personality changes, dementia and suicidal tendencies (Bhadra *et al.* 2013).

T. gondii has subpopulation structures in different geographical regions, which formed by less frequent sexual recombination, population sweeps and biogeography (Sibley *et al.* 2009). The majority of strains isolated in North America and Europe fall into one of three clonal lineages, referred to as types I, II and III (Howe & Sibley, 1995). Lately, studies suggest the fourth clonal lineage is commonly found in wildlife in North America (Khan *et al.* 2011). Clonality is associated with the recent emergence of a monomorphic version of Chr1a, which drove a selective genetic sweep within the past 10,000 years (Khan *et al.* 2007, Sibley *et al.* 2009). However, in South America these three strains are sporadically isolated and instead distinct strains with a great genetic diversity are more popular (Ajzenberg *et al.* 2004). Recently, a hallmark study examining the genetic diversity of *T. gondii* was conducted on ~950 isolates collected from around the world (Su *et al.* 2012). Using three genotyping methods, 15 haplogroups that define 6 major clades have been revealed. These haplogroups comprise the 3 main clonal lineages widely spread in North America and Europe, various atypical genotypes found in South America and new clonal lineages. Recent research support the concept that many atypical genotypes differ in pathogenicity and transmissibility from typical genotypes (Darde 2008, Maubon *et al.* 2008, Carme *et al.* 2009, Lindsay & Dubey 2011).

The population structure of *T. gondii* in North America and Europe is well defined. The three clonal lineages exhibit low level of genetic divergence (only 1~2% divergence at the DNA sequence level). However, they have remarkably different virulence phenotypes in mice, with type I strains are uniformly lethal in mice but type II and III strains are considerably less virulent. In addition, type I strain display enhanced migratory capacity *in vitro* and *in vivo*, faster growth rate *in vitro*, and reach higher parasite loads in laboratory mice (Saeij *et al.* 2005). Pathogenicity differences among the three strains are greatly determined by polymorphism and expression differences in proteins such as dense granule and rhoptry (e.g. GRA15, ROP5, ROP16, and ROP18) (Melo *et al.* 2011). The genotypes not belonging to the three main lineages were found predominant in other continents where

the population structure of *T. gondii* was more complex, with a high genetic diversity (Darde 2008). They possess a shuffled combination of alleles that typify the three clonal types and unique polymorphisms, indicating they have a more ancient origin (Khan *et al.* 2005). These strains range from the highly virulent to the intermediate or non-virulent phenotype in mice (Darde 2008). However, relatively little is known regarding effectors of these atypical strains.

Given the clearly different population structure of *T. gondii* in the world, it is important to address the significance of this diversity on human disease. In this review, we first focus on methods for *T. gondii* strain typing, and then present an overview of infecting *T. gondii* strain on different disease manifestations in humans with some considerations for possible mechanisms. Before describing these data, it is critical to point out that the term "virulent" or "avirulent" strain used in this paper is based on the definition of virulence in outbread mice. However, mouse virulence may not apply to all hosts, e.g. rats are highly resistant to *T. gondii*. Recently, scientists in Colombia have attempted to correlate severity of clinical toxoplasmosis in patients with strain-specific effector isoforms identified in mice. They found the severe ocular inflammation in ocular toxoplasmosis is associated with infection by strains harboring the ROP18 type I allele (Sanchez *et al.* 2014).

Methods for *T. gondii* strain typing

We discussed genetic and serological discriminatory methods that have been widely used in the past decade, and their relative advantages and disadvantages (Fig. 1). Genotyping offers high sensitivity and specificity which is a reliable approach for determining *T. gondii* genotype. However, this technique requires obtaining parasite DNA from infected host. Although roughly 25~30% of the worldwide human population is infected by *T. gondii*, the most frequent form of infection is latent in which parasites are usually not found in circulation and obtain organisms are extremely difficult (Robert-Gangneux & Darde, 2012). As *T. gondii* can induces a strong and often persistent humoral immune response with detectable antibody titers, independent of the clinical manifestations in the infected individuals (Parmley *et al.* 1994, Dubey 2008), this problem has been partially alleviated by the development of serological tests that detect epitope-specific antibody responses.

Methods for genotyping

Genotyping employing PCR-based molecular analyses have been successfully applied in various clinical samples. As has been reviewed in detail by Su *et al.* (2010), several methods including restriction fragment length polymorphism (RFLP)-PCR analysis, sequence length polymorphism-based microsatellite analysis, and multilocus sequenced-based analysis are commonly used, each with its advantages and limitations. In most cases, the sensitivity of specific detection of the assay is of primary concern, which highlights the necessity of using multiple markers to achieve high resolution (Su *et al.* 2006, Ajzenberg *et al.* 2010).

Multilocus PCR-RFLP genotyping is the better choice to distinguish the major clonal lineages from atypical strains. The method is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), digest PCR products and subsequently display distinct DNA banding patterns on agarose gels by electrophoresis

(Su *et al.* 2010). Howe and Sibley (1995) have conducted a hallmark study to determine the population genetic structure of *T. gondii* employing 106 isolates originated from Europe and North America. Surprisingly, only 3 predominant lineages (types I, II and III) were identified by using 6 PCR-RFLP markers, which lead to the conclusion that *T. gondii* has a highly clonal population structure. However, the PCR-RFLP method depends on single-copy polymorphic DNA sequences, which could result in a compromised sensitivity (estimated at ≥100 *T. gondii* genome equivalents) (Su *et al.* 2010). Another disadvantage is that RFLP markers are restricted to capturing changes occurred at restriction enzyme sites; as a result, many polymorphisms at other loci are missed (Sibley *et al.* 2009).

Microsatellite analysis (MS) is especially useful in revealing very recent mutations in closely related isolates within a lineage, as the rates of mutation of microsatellites (10^{-2}–10^{-5} per locus per replication) are much higher compared with that of point mutations in RFLP markers (10^{-9}–10^{-10}) (Goldstein & Schlotterer, 1999). MS is based on short tandem repeats of between two and six nucleotides. Their polymorphism is caused by different numbers of repeats in alleles, which result in variable lengths (Blackston *et al.* 2001, Ajzenberg *et al.* 2004). These sequences are highly polymorphic which provide enormous information in genetic and epidemiological researches. Ajzenberg *et al.* (2010) demonstrated the utility of employing multiple microsatellite markers for characterization of *T. gondii* genotypes at both the typing (types I, II, and III versus atypical strains) and fingerprinting levels (closely related isolates within a clonal lineage). There are several limitations of this assay: 1) the relatively low sensitivity, estimated at 50 to 100 *T. gondii* genome equivalents per 5 μl of DNA sample (Ajzenberg *et al.* 2010); 2) these MS markers are vulnerable to homoplasy as the number of repeats can expand and contract during replication (Sibley *et al.* 2009).

The multilocus sequence typing (MLST) is based on polymorphic variation in DNA sequences comprising the SNPs, insertion and deletion of nucleotides (Su *et al.* 2010). DNA sequencing has allowed detailed and systematic characterization of specific genome fragments (Lyons & Johnson, 1998,, Fazaeli *et al.* 2000, Aspinall *et al.* 2003). Although Multilocus PCR-RFLP and MS markers detect genetic variation, they do not reveal all polymorphisms present in a given locus. For example, a total of nine different alleles from 30 strains were identified at the GRA6 locus by sequencing, while only three groups was detected by PCR-RFLP method (Fazaeli *et al.* 2000). Recent studies using this approach have showed that the genetic makeup of *T. gondii* in Brazil is more complex than previously recognized with the presence of unique genotypes (Khan *et al.*, 2006, 2007). Thus, sequence-based analysis is superior to all other typing methods because it provides the complete genetic polymorphisms of genomic regions. However, the increased cost and investment of time makes this method unsuitable for genetic screening of a large number of isolates (Ajzenberg *et al.* 2010). In fact, this method is particularly suitable in new isolates or from previously unsampled populations (Sibley *et al.* 2009).

Methods for serotyping

Serotyping is based on the observation that the three clonal lineages of *T. gondii* differ not only genetically but also in the amino acid sequences of several parasite proteins, leading to

polymorphic sites (Parmley *et al.* 1994, Maksimov *et al.* 2012). Humoral responses to these polymorphic sites are thus strain specific (Parmley *et al.* 1994, Kong *et al.* 2003). Serotypes are defined by the antibody profiles against these polymorphic sites. Serotyping is a very appealing approach because this technique avoids isolating parasite from human samples which allows extending typing studies to the vast majority of infected people where *T. gondii* causes little if any disease.

The dense granules (GRA) are secretory organelles that responsible for cell invasion and intracellular survival of this apicomplexa parasite. GRA proteins are expressed by the three stages of *T. gondii*: the tachyzoite, bradyzoite, and sporozoite (Sousa *et al.* 2009). Their immunogenic and polymorphic properties make these antigens suitable for serotyping studies (Fazaeli *et al.* 2000, Pietkiewicz *et al.* 2007, Sousa *et al.* 2009). For example, compared to peptides derived from surface antigens and rhoptry proteins, peptides derived from GRA proteins had more robust reaction when tested with human sera (Maksimov *et al.* 2012). Based on polymorphic sequences (Fazaeli *et al.* 2000, Zakimi *et al.* 2006, Sousa *et al.* 2009), a series of GRA peptides derived from multiple loci (e. g. GRA3, GRA5, GRA6 and GRA7) are commonly used to identify the clonal type of humans or animal infected with. Moreover, Vaudaux *et al*. (2010) have demonstrated the usefulness of these archetypal peptides in identifying nonarchetypal strains. In the absence of parasite DNA from individuals infected during the Santa Isabel outbreak, serotyping was performed using GRA6 and GRA7 peptides to characterize the exposure (Vaudaux *et al.* 2010). Serotyping successfully discovered that the outbreak was caused by an atypical, clonal, and the dominant type retrieved among animals at the same time.

The pioneering work of Kong *et al.* (2003) demonstrated that synthetic peptides from polymorphic antigens have the potential to predict the clonal type of humans or mice infected with. Since then, several efforts have been made towards serological typing *T. gondii* infection using ELISA formats. Some researchers have linked synthetic peptides to the carrier protein KLH (Kong *et al.* 2003, Nowakowska *et al.* 2006), while others directly coated the peptides to the solid phase (Sousa *et al.* 2008, 2009, 2010, Xiao *et al.* 2009). In addition, serotyping using recombinant antigens or synthetic peptide-microarray was also tested (Peyron *et al.* 2006, Morisset *et al.* 2008, Maksimov *et al.* 2012, 2013). These assays suggested that it is possible to distinguish between type II- and non-type II-infections (Kong *et al.* 2003, Peyron *et al.* 2006, Sousa *et al.* 2008, Maksimov *et al.* 2012, Maksimov *et al.* 2013), while it is extremely difficult to distinguish type I from type III infections in that they share more identical alleles at many loci (Kong et al. 2003). However, Xiao *et al.* (2009) have identified peptides to distinguish type III- from type I-infections. The authors have developed a two-step screening of serotyping: the first-step is to distinguish type II infection from type I/III infection using five peptides (3 type II peptides and 2 type I/III peptides); the second-step is to distinguish type III infection from type I infection using GRA7-III peptide. In agreement with Xiao's finding, Sousa and colleagues (2009) suggested that peptide originated from GRA7III might be a good candidate for the serotyping of infections caused by type III strains based on large numbers of sequencing results on GRA7 antigens.

Although current serotyping approaches offer robust results, they do possess significant limitations. First, they lack the ability to unequivocally determine all three lineages. The

primary reason is because the three clonal types may have arisen from common ancestors of two closely related but genetically different lineages, many of the polymorphic sites are specific for more than one of the three clonal types I, II or III (Maksimov *et al.* 2012). Second, a proportion of sera displayed no reaction with these polymorphic peptides (Kong *et al.* 2003, Xiao *et al.* 2009). Third, these tests are limited to distinguishing within all three lineages (Sousa *et al.* 2008, Xiao *et al.* 2009), and they do not have sufficient diversity to accurately type strains with distinct genetic structure, such as strains isolated in South America, Africa and Asia. To overcome these problems, well-characterized reference sera and highly specific polymorphic peptides are urgently needed.

A possible link between human disease and *T. gondii* strain

In humans, data of infecting strain types exist for four main groups: ocular infection in immunocompetent adults; congenital infection in the fetus or newborn; adults with AIDS or other immunocompromised states; and severe diseases (e.g. disseminated, pulmonary toxoplasmosis and psychosis) in immunocompetent patients. For this review, we excluded studies that employed a single genetic marker for strain determination as this may lead to a misleading genotype designation in some instances. Overall, the presence of particular *T. gondii* strains is under the influence of patients' geographic origins, but virulent strains containing type I or atypical alleles are more pathogenic or more likely to cause severe disease in patients. Figure 2 summarized the impact of *T. gondii* strains on the severity of human diseases.

T. gondii strain type and ocular toxoplasmosis

Ocular toxoplasmosis (OT) is a potential complication of both acquired and congenital infection, leading to visual impairment in numerous countries and being responsible for 30 to 50% of uveitis cases in immunocompetent individuals (de-la-Torre *et al.* 2013).

The prevalence of ocular involvement among *T. gondii*–infected individuals varies geographically. For example, OT in the United States and Europe is approximately 2%, compared to nearly 18% in southern Brazil (Glasner *et al.* 1992, Holland 2003). In Minas Gerais, Brazil, the presence of retinochoroidal lesions in congenital toxoplasmosis (CT) was 79.8% (Vasconcelos-Santos *et al.* 2009). Moreover, the clinical manifestation in South America is different with those reported in Europe. A comparative prospective cohort study of congenitally infected children (Gilbert *et al.* 2008) revealed there was a five-times higher risk of developing eye lesions in Brazilian children when compared to European children; in addition their lesions were larger, more multifocal, more recurrent and more likely to impair vision. Two-thirds of Brazilian children infected with CT had eye lesions by 4 years of age compared with one in six in Europe. These differences have been attributed to atypical or recombinant genotypes circulating in South America (Khan *et al.* 2006, Gilbert *et al.* 2008). In support of this, de-la-Torre *et al.* (2013) demonstrated in a cross sectional study that Colombian OT patients were infected by type I or atypical strain, whereas the French were uniformly possessed type II serotypes. Among the 17 criteria analyzed in the two populations, the following were significantly higher in Colombian patients: macular involvement, vitreous inflammation, strabismus, bilateral involvement and synechiae.

Studies centered in North America and Europe revealed a conspicuous bias toward non-type II strains associated with severe OT in humans. In a small series of patients with severe ocular inflammation from the United States, there was an unusual abundance of type I or atypical parasites (Grigg *et al.* 2001). In German uveitis patients, a novel, nonreactive (NR) serotype was significantly more frequently in sera of OT patients than non-OT patients ($p < .0001$). Among OT patients, those with NR serotypes experienced more frequent recurrences ($p = .037$) (Shobab *et al.* 2013). Nonarchetypal strain infection provide an explanation for why the NR serotype does not produce antibodies against archetypal peptides, because nonarchetypal strains could either possess entirely different polymorphic epitopes at GRA6 or GRA7 or lack of immunogenicity at these polymorphic epitopes. In ocular samples collected from 20 French patients, Fekkar *et al.* (2011) reported a predominance of type II genotype. Considering the local epidemiological data, the authors suggested that the prevalence of type II infections in OT may be due to more common exposure to strains of this genotype. Notably, atypical strain was also identified in two of four French OT patients (Xiao *et al.* 2013b).

The correlation between ocular disease and parasite genotype is further reinforced by differences in the incidence of ocular involvement in different outbreaks of acquired toxoplasmosis. In 2001, a large toxoplasmosis outbreak occurred at the town of Santa Isabel do Ivai, Parana state, Brazil. This outbreak affected at least 426 people and was characterized by a high prevalence of symptomatic, systemic disease (de Moura *et al.* 2006) and ocular involvement (Vaudaux *et al.* 2010). By serotyping, an atypical strain was found to be responsible for the outbreak (Vaudaux *et al.* 2010). In an outbreak of acute toxoplasmosis occurred in Atlanta in 1977, only 1 (3.6%) of 28 clinically symptomatic people developed ocular disease (Akstein *et al.* 1982). However, in another outbreak occurred in 1995 in Victoria, Canada, 20 (20.6%) of 97 people who experienced symptomatic infections in that epidemic developed ocular disease, a significantly greater proportion of incidence (Burnett *et al.* 1998). Although the strain responsible for the Atlanta outbreak is still unknown, the strain isolated from a patient in the Victoria outbreak was confirmed as a type I strain (Lehmann *et al.* 2000).

T. gondii strain type and congenital toxoplasmosis

Congenital infection can cause pregnancy loss (miscarriage or stillbirth) or severe disease in the newborn, including developmental delays, blindness, and epilepsy (Jones *et al.* 2014). However, a majority of newborns with CT are asymptomatic at birth. Even if asymptomatic at birth, illness will develop in many infected infants later in their life, most often associated with ocular disease, but also neurologic symptoms and developmental disabilities (Jones *et al.* 2014). The principal factor determining the severity of CT is the gestational age at the time of fetal infection, with infection in early pregnancy displaying more severe consequences than infection in late pregnancy, but the parasite genotype may also play a role (Ajzenberg *et al.* 2002, Delhaes *et al.* 2010a).

In several studies centered in Europe, CT was triggered more frequently by type II strains (Ajzenberg *et al.* 2002, Nowakowska *et al.* 2006). Ajzenberg *et al.* (2002) analyzed 86 *T. gondii* isolates collected from patients with CT in France. They noticed atypical genotypes

are extremely rare in France where the predominant isolates were type II. The type II strain has caused severe symptoms in some individuals and milder ones in others. The outcome between asymptomatic and severe CT caused by type II strains is strongly related to the time of gestation when maternal infection occurs: infection in early pregnancy with type II strains results in more severe outcome than infection in later pregnancy with type II strains, following the general rule (Ajzenberg *et al.* 2002, Delhaes *et al.* 2010a). Among the 86 patients, 45 were the subclinical or benign cases in which no type I or atypical genotypes were observed. These findings seem suggest that type II strain is not a risk factor for severity of congenital infection.

A few severe cases of CT reported in France were due to infection with atypical *T. gondii* genotypes (Ajzenberg 2012). For example, an atypical genotype, determined from the amniotic fluid of a pregnant woman who was native to France, was reported to result in severe CT with bilateral ventricular enlargement and calcifications (Delhaes *et al.* 2010a). Pomares *et al.* (2011) described 3 severe cases of toxoplasmosis (2 CT and 1 disseminated toxoplasmosis) caused by atypical strains in France. Because atypical strains are unusual in France, the authors suspected that the infecting strains of these patients probably obtained by consumption of imported horse meat. Delhaes *et al*. (2010a) reviewed the literatures reporting CT cases caused by maternal infection with an atypical strain. They observed that most of CT cases due to atypical strains have poor outcome regardless of therapy management (Delhaes *et al.* 2010a). Moreover, severe CT is rare when maternal infection occurred at the beginning of the third trimester and was never observed with type II strains in 86 patients with CT in France (Ajzenberg *et al.* 2002, Delhaes *et al.* 2010a). In contrast, atypical strains obtained at the third trimester were able to cause severe consequence in offspring (Delhaes *et al.* 2010a). Incidentally, in the few cases of congenital infection in infants born to mothers with preexisting immunity to *T. gondii*, re-infection by an atypical strain was suspected to be the underlying cause (Elbez-Rubinstein *et al.* 2009).

Contrary to what is observed in France, studies from the United States indicate that type II strains are not predominant in CT. McLeod *et al.* (2012) conducted a study where parasite serotype was determined for 193 congenitally infected infants and their mothers in the NCCCTS, 1981–2009. The authors noted that NE-II (all non–type II) serotypes are more often present than type II serotypes in infants with CT (61% vs 39%) in the United States (McLeod *et al.* 2012). Moreover, infants with NE-II serotypes are more likely to experience severe disease and eye severity at birth than those with type II serotypes. Previously, findings in a study of 106 isolates with different origins of isolation from North America and Europe suggested that type I isolates were significantly more often associated with CT than with animal infection or with reactivation of chronic infections in patients with AIDS (Howe & Sibley 1995).

In South America, CT is more often associated with severe symptoms, as usually a result of infection with atypical strains. Gilbert *et al*. (2008) reported that Brazilian infants with CT have more severe ocular disease compared to European counterparts. Carneiro *et al.* (2013) revealed the genetic diversity of *T. gondii* isolates from newborns with CT in Southeastern Brazil. They reported a total of 14 different genotypes in which most of them are

intermediately or highly virulent to mice. Moreover, of the 24 isolates completely genotyped, 20 originated from newborns with retinochoroiditis.

There are limited studies reporting the strain of infecting parasite in congenital infection from other geographical origins. In Turkey, Africa 1 genotype was identified in two cases of CT (Doskaya *et al.* 2013). Africa 1 has similar clonal and virulence properties with the BrI strain (atypical) isolated from Brazil. In Tunisia, North Africa, type I recombinant strains are found to be overrepresented in congenital infection (Boughattas *et al.* 2010). Human infection with the recombinant strain can result in serious consequences during congenital transmission (Boughattas *et al.* 2011).

T. gondii strain type in immunocompromised patients

In immunocompromised patients, *T. gondii* is a major opportunistic pathogen that may cause life-threatening disease, as it most often involves the central nervous system and symptoms may include those of meningoencephalitis or mass lesion such as headache, confusion, fever, lethargy, seizures and focal neurological signs. In patients with AIDS, toxoplasmosis mainly occurs after reactivation of latent infection, rarely during a primary infection (Ajzenberg *et al.* 2009).

In studies centered in North America and Europe, type II was the predominant strain in immunocompromised patients, but this may simply reflect the prevalence of type II infection in general population. As early as 1995, Howe and Sibley compared the genotypes of *T. gondii* strains associated with animal versus human infections and noted most infections in AIDS patients were caused by type II strains. In a genotyping analysis performed on isolates collected from 88 immunocompromised patients, type II strains were predominant among patients who obtained toxoplasmic infection in Europe (Ajzenberg *et al.* 2009). However, the distribution of type II vs non-type II strains was not significantly different when patients were stratified by underlying cause of immunosuppression, site of infection (cerebral or extra-cerebral), or outcome. Non-type II isolates were also identified in this study but mainly collected from patients who obtained toxoplasmosis outside Europe, e.g. Africa 1 genotype was detected in nine immune-compromised patients who were mostly from sub-Saharan Africa (Ajzenberg *et al.* 2009). These results suggest that the genotype of *T. gondii* strains is strongly linked to the geographical origin of infection.

Studies have suggested that type I strains are more pathogenic in immunocompromised patients. In an effort to identify the genotypes of *T. gondii* associated with immunocompromised patients, Khan et al (2005) analyzed 11 cerebral spinal fluid (CSF) samples collected during the period of 1990 to 1996. All of the patients had confirmed or presumptive TE. They found a majority of these patients had infections with type I strains or strains containing type I alleles. Ferreira *et al.* (2008) investigated the genotypes of *T. gondii* strains isolated from 87 patients with cerebral toxoplasmosis and AIDS, treated in Sao Paulo State, Brazil. Although their study revealed a high rate of genetic polymorphism in *T. gondii* strains isolated in Brazil, type I seems to be prevalent as this strain was responsible for infection in 46% of their patients (Ferreira *et al.* 2008).

Several studies reported an increased clinical severity of toxoplasmosis due to infection with atypical and/or recombinant strain in the setting of immunosuppression. Genot *et al.* (2007) reported a case of severe encephalitis as a consequence of reactivation *T. gondii* I/III recombinant genotype in an African HIV patient. Ghosn *et al.* (2003) described a case of toxoplasmic adenitis in an HIV-infected patient with a past history of TE, after discontinuation of secondary prophylaxis due to considerable immune recovery. This manifestation was suspected as a result of reinfection with an atypical strain of *T. gondii*, as the patient stayed in the French West Indies for long time. Delhaes *et al.* (2010b) reported a severe pulmonary toxoplasmosis in two HSCT (Hematopoietic stem cell transplantation) patients due to infection with atypical and type III strains, respectively. Štajner *et al.* (2013) reported a fatal outcome caused by reactivation of an atypical strain in a HSCT recipient who developed an early and fulminant toxoplasmosis. In contrast, Patrat-Delon *et al.* (2010) presented a favorable outcome in a heart transplant recipient with disseminated toxoplasmosis where the reactivation was caused by type II.

T. gondii strain type and severe diseases

Recent researches shed light on new clinical aspects of *T. gondii* infection in immunocompetent people, such as the severe outcome caused by strains from the Amazon rainforest and the involvement of *T. gondii* in brain disorders. These new characteristics have broadened our vision on the pathogenicity of *T. gondii* infection.

Since *T. gondii* has a privileged interaction with the CNS, the hypothesis that *T. gondii* infection could increase the individual's susceptibility to many brain disorders, especially schizophrenia, was proposed (Torrey & Yolken, 2003). A meta-analysis of 38 studies of *T. gondii* seropositivity in individuals with schizophrenia compared to controls reported an odds ratio of 2.73 (Torrey *et al.* 2012). Moreover, *in vitro* observation showed that several antipsychotic drugs commonly used for treating schizophrenia could inhibit *T. gondii* replication (Jones-Brando *et al.* 2003). There are several plausible mechanisms by which *T. gondii* could cause schizophrenia. First, *T. gondii* has the ability to produce dopamine (Gaskell *et al.* 2009) and increased levels of dopamine and its metabolites have been observed both *in vitro* and *in vivo* (Prandovszky *et al.* 2011, Xiao *et al.* 2014). Secondly, *T. gondii* could affect host (mouse and human cells) production of GABA, glutamate, and serotonin with differential effects depending on the strain of the parasite (Fuks *et al.* 2012, Xiao *et al.* 2013a). Thirdly, the immune response to *T. gondii* could affect cytokine expression (Bhadra *et al.* 2013) and kynurenic acid pathway (Schwarcz & Hunter 2007), both of which have been indicated in the pathogenesis of schizophrenia. Lastly, *T. gondii* may not cause symptoms directly but rather by interacting with other genetic susceptibility factors and/or environmental insults (Torrey & Yolken 2014).

Evidence from serotyping indicated that type I strain was involved more in patients with psychiatric disorders (Xiao *et al.* 2009, Groer *et al.* 2011). In a study from the United States, Xiao *et al.* (2009) had identified serotypes of *T. gondii* in 219 pregnant women whose children developed schizophrenia and psychotic illnesses in adult life and 618 matched unaffected control mothers. They found that the offspring of mothers with *T. gondii* type I infection were at significantly increased risk for the development of psychoses as compared

with the matched unaffected control mothers. There was no significant association between other serotypes of *T. gondii* and risk of psychoses in adult offspring. Groër *et al.* (2011) also found that *T. gondii*–infected pregnant women are at risk for the dysphoric mood states of depression and anxiety. The depression and anxiety scores were highest among pregnant women infected with *T. gondii* type I serotype, although this did not reach statistical significance.

Studies suggest that strains originated from French Guiana can lead to severe disease and even death in immunocompetent people (Darde *et al.* 1998, Bossi *et al.* 2002, Carme *et al.* 2002, Groh *et al.* 2012). From 1998 through 2006, 44 cases of severe primary toxoplasmosis were recorded (Carme *et al.* 2009). All patients had been hospitalized and about one third of them experience respiratory distress and need to be in an intensive care unit (Carme *et al.* 2009). Genotyping analysis showed that *T. gondii* strains isolated from these patients exhibited an atypical multilocus genotype. Demar *et al.* (2007) described a community outbreak of multivisceral toxoplasmosis that occurred in Patam, a Surinamese village near the French Guianan border. From late December 2003 through mid-January 2004, 11 cases occurred among 33 inhabitants of a Surinamese village (Demar *et al.* 2007). Among the 11 cases, eight immune-competent adults showed multi-visceral toxoplasmosis, leading to one death; one neonate and one fetus had lethal CT; and one child had symptomatic toxoplasmosis. Using microsatellite markers, the authors identified that one atypical strain was responsible for this outbreak. Lately, Demar *et al.* (2012) described a disseminated toxoplasmosis with life-threatening pneumonia, occurring in 11 immunocompetent adults at French Guianese from 2002 to 2008. Each patient required intensive care management, as they displayed at least one form of organ failure (Demar *et al.* 2012). Genotyping analysis revealed atypical and unique multilocus genotypes from eight patients. These severe forms caused by atypical strains were also reported in European people consuming meat originated from Brazil (Pomares *et al.* 2011, Sobanski *et al.* 2013).

Possible mechanisms

Several hypotheses, other than human immune status, have been suggested to explain the differential pathogenicity of *T. gondii* genotype in humans, such as strain-related differences in pathogenicity, poor host (human) adaption, and human genetic predisposition (Suzuki *et al.* 1996, Romand *et al.* 2004, Carme *et al.* 2009).

Strain-related differences in pathogenicity

Several studies reported the severity of toxoplasmosis in immunocompromised patients is associated with parasite burden which depending on the strain of the parasite. Genot *et al.* (2007) described a high parasite density in the brain and CSF of a HIV patient infected with a type I/III recombinant genotype, who had unusually severe encephalitis and chorioretinitis associated with a cerebral salt wasting syndrome. In the case of fatal reactivation of an atypical *T. gondii* strain in a HSCT recipient with underlying immunological deficiency, the extremely high parasite burden in blood and BAL fluid was observed (Stajner *et al.* 2013). In contrast, the favorable outcome of type II reactivation in a heart transplant patient was correlated with a much low parasite load (Patrat-Delon *et al.* 2010).

Studies from congenital and ocular infections indicate the presence of *T. gondii* positivity in samples was associated with an increased clinical severity. In a recent Brazilian study (Costa *et al.* 2013), *T. gondii* qPCR positivity in blood samples of newborns with CT was associated with the presence of retinochoroidal lesions. Among infants with active retinochoroiditis, 68% had positive qPCR results, while positivity was much lower (29%) in the absence of ocular involvement (Costa *et al.* 2013). Interestingly, *in vitro* studies have shown that both RH and South American strains are able to access human retinal endothelium from the circulation in tachyzoite form, either unassisted (Furtado *et al.* 2012b) or in a leukocyte taxi (Furtado *et al.* 2012a). In a study from German patients with OT (Shobab *et al.* 2013), type II-infected patients generally do not possess sufficient parasite DNA in aqueous humor to permit genotyping. The lack of detectable parasites was positively correlated with the decreased numbers of recurrences among this patient cohort (Shobab *et al.* 2013). Thus, it is hardly surprising that parasitic burden determined by qPCR may influence the choice of treatment (Kupferschmidt *et al.* 2001, Martino *et al.* 2005).

There are some evidence supporting a possible correlation between parasite load in amniotic fluid and fetal outcome or clinical status at birth (Romand *et al.* 2004, Yamada *et al.* 2011). Romand *et al.* (2004) also indicated that the parasite burden in amniotic fluid is an independent risk factor for fetal outcome, in addition to the gestational age at maternal seroconversion. Specifically, maternal infections obtained before 20 weeks with a parasite load larger than 100/mL of amniotic fluid have the highest risk of severe fetal outcome (Romand *et al.* 2004). Conversely, the majority of cases with a parasite concentration below 100/ml of amniotic fluid are mild or subclinical. The authors suggested that an assessment of the concentration of parasites in amniotic fluid may serve as a prognostic tool in an attempt to predict the clinical course of congenital infection. Similarly, Kieffer *et al.* (2011) reported a case of disseminated CT due to infection with a type II strain at the third trimester. Quantitative PCR showed high parasite loads on various specimens, with parasite concentrations to a maximum of 3800/mL (Kieffer *et al.* 2011), a fact that could explain the poor outcome.

Generally, infection with *T. gondii* can provide the host with lifelong protective immunity against reinfection. However, several reports confirmed that acquired immunity against clonal types may not protect against reinfection by atypical strains (Elbez-Rubinstein *et al.* 2009). In this regard, Elbez-Rubinstein *et al.* (2009) confirmed that acquired immunity against type II strains may not protect against reinfection by atypical strains. Similarly, HAART-induced immune recovery of *T. gondii* infection may not be protective against reinfection with atypical strains, as Ghosn *et al.* (2003) have reported. Ferreira *et al.* (2008) also noticed re-infections in some HIV patients with cerebral toxoplasmosis in Brazil (where most clinical isolates are atypical or recombinant) because slightly different genotypes of *T. gondii* strains have been found in the same patients. Using an experimental model, the capacity of atypical strains to re-infect mice with past type II infection and to produce cysts that coexist with type II cysts has been confirmed (Elbez-Rubinstein *et al.* 2009).

In vitro studies have documented a distinct host response to different *T. gondii* strains (Xiao *et al.* 2011, 2013a). Xiao *et al.* (2011) examined the transcriptional profile of human neuroepithelioma cells in response to the three typical genotypes using microarray analysis.

Neuroepithelioma cells infected by type I exhibited the highest level of differential gene expression whose function largely related to the central nervous system. Cells infected by type II had a smaller number of gene expression and did not alter specific gene pathway. Type III infection had intermediate effects on gene expression and largely altered genes involved in nucleotide metabolism. When further analyzing the neurotransmitter and neuropeptide systems of the infected cells, Xiao *et al.* (2013a) noted that type I infection caused abnormalities in three neurotransmitter receptors (dopamine, glutamate and serotonin) and two neuropeptides (PROK2 and TAC1), while type III infection led to the change of a critical enzyme (TDO2) in the kynurenine pathway. In contrast, no significant effects of type II infection were found.

Several studies reported apoptosis in human trophoblastic cells during *T. gondii* infection is associated with the virulence characteristics of the parasite. Angeloni *et al.* (2009) noted that BeWo choriocarcinoma cells infected with type II strain presented higher apoptosis than type I-infected cells. To explore the factors involved in the differential apoptosis, Angeloni *et al.* (2013) investigated the profile of cytokines secreted by BeWo cells infected with type I or II strains of *T. gondii*. Interestingly, type II-infected BeWo cells exhibited a predominantly pro-inflammatory cytokine profile, with higher secretion of MIF, TNF-a, IL-12, IL-17A and IL-6. In contrast, type I-infected cells had a higher production of anti-inflammatory cytokines including TGF-b1 and IL-10 (Angeloni *et al.* 2013). Their results indicate that type I and II strains of *T. gondii* possess opposing mechanism of interference in apoptosis: avirulent type II strain elicited an effective mechanism directed at alerting and activating host's immune system, while virulent type I strain employed strategy to evade of the immune response (Angeloni *et al.* 2009). Since the increased apoptosis might result in reduced parasitemia, type II strain showed a protective mechanism for the host. Such differences can be critical for the outcome of infection in CT. Another study compared three typical strains in their capacity to infect placental interfaces using first-trimester human placental explants (Robbins *et al.* 2012). No significant difference was observed but type II strain showed a slightly slower replication rate.

Host (human) adaptation

Since the genetic diversity is relatively limited in European *T. gondii* strains, a long history of host (human) adaptation and human–parasite co-evolution involving more or less the same strain may explain why toxoplasmosis usually has no clinical consequences in immunocompetent individuals in Europe (Ajzenberg 2011). In contrast, the highly virulent strains emerged from the forest-based cycle involving wild felids and their preys in French Guiana, suggesting that the parasite and humans have not adapted to each other (Carme *et al.* 2009). Severe disease in humans has thus been speculated that may result from poor host adaptation to exotic strains (Carme *et al.* 2009). Several studies gathered some evidence supporting this hypothesis.

The strain-specific differences in how *T. gondii* stimulates host's innate immunity are probably responsible for, at least in part, the distinct pathogenicity caused by these parasites during infection. Previously, Yamamoto *et al.* (2000) observed that asymptomatic persons had high levels of IL-12 and IFNγ in response to *T. gondii* antigens than patients with ocular

lesion. Ongkosuwito *et al.* (1998) evaluated the T-helper cell cytokine profiles directly in ocular fluid samples from patients with viral or toxoplasmic uveitis. Among patients with toxoplasma chorioretinitis, IL-6 levels were significantly higher in patients with active disease than patients with nonactive disease. As the population structure of *T. gondii* in Europe and South America are so different, a comparative study to examine clinical, parasitological and immunological responses and to relate them with the infecting strains has been conducted (de-la-Torre *et al.* 2013). In French patients, the host-parasite relationship seems to be equilibrated between protection and inflammation. The protective effect of IFNγ is balanced by anti-inflammatory cytokines such as IL-2 and IL-10. Such cytokines may lead to the encystation of the parasite in the retinal tissues. In contrast, in the clinically more severe Colombian cases, IFNγ and other major immunomodulators such as IL-17 were barely detectable, while IL-6 and IL-13 were enhanced. Colombian patients display a more suppressive immune response which account for the drastically higher local parasite proliferation. Further work from Colombian patients documented that the particular severity of ocular toxoplasmic infection is due to a predominant Th2 response thus preventing effective parasite control (de-la-Torre *et al.* 2014). In support of these findings, virulent type I strains are known to inhibit IL12 production, a major determinant of Th1 response, in human fibroblast cell lines (Saeij *et al.* 2007). Another study reported that *T. gondii* seems to strain-specifically impact levels of circulating cytokines in pregnant women (Pernas *et al.* 2014). In this regard, a large number of cytokines (n = 23) were at lower levels in the patients from US (approximately one-half of clinical isolates are type II), while only a small number of cytokines (n = 4) were at lower levels in Colombian cohort (generally “atypical” or type I-like strains) (Pernas *et al.* 2014).

Similarly, *in vitro* studies indicate the type of immune response elicited by *T. gondii* depends on the strain type. A recent study showed strain-specific differences in the expression of pro-inflammatory protein among different human nervous cells (Mammari *et al.* 2014). Type II strain, but not type I, stimulates all cells to produce pro-inflammatory growth factors, G-CSF and GM-CSF. These proteins could increase the inflammatory effect of this type II strain (Mammari *et al.* 2014). Xiao *et al.* (2011) noticed an inhibition on pro-inflammatory genes such as IL-8 in human neuroepithelioma cells infected by type I. A recent study in human monocytes provides evidence that the production of IL-1β, a key regulator of inflammation and innate immunity, is strain dependent (Gov *et al.* 2013). Among the type I, II, and III strains of *T. gondii*, the type II strain induced substantially more IL-1β mRNA and protein release than did the type I and III strains (Gov *et al.* 2013). In agreement with these findings are results from *T. gondii* modulates the NF-kB pathway, as IL-1β transcript is known to be induced by downstream of NF-kB signaling (Rosowski *et al.* 2011, Gov *et al.* 2013). Rosowski *et al.* (2011) demonstrated that type II strains activate more NF-kB than type I or type III strains. The polymorphic protein GRA15 which *T. gondii* secretes into the host cells upon invasion is responsible for these differential effects (Rosowski *et al.* 2011, Gov *et al.* 2013). Morampudi *et al.* (2011) observed a strain-specific difference in activation of early innate mechanisms in human intestinal epithelial cells (IEC). They observed that type I parasites induce poor early innate immunity in human IEC, which includes a failure to induce HBD2. In contrast, type II and III parasites induced the

early expression of HBD2. These results suggest that host (human) cells did not exert a strong protective effect against type I infection.

Genetic susceptibility of the human

Studies indicate that the strain of *T. gondii* is not sufficient to predict outcomes in human infection and the severity of toxoplasmosis is strictly contextual. For instance, inter-individual variabilities (some patients died or were seriously ill whereas other patients only have mild symptoms) have been observed in an outbreak caused by a same Guianan strain (Demar *et al.* 2007). Another study from France in immunocompromised patients also suggested that host factors are largely involved in patients' resistance or susceptibility to toxoplasmosis (Ajzenberg *et al.* 2009).

The human leukocyte antigen (HLA) system is one of the most polymorphic genetic systems in humans and has important functions in combating both intra-and extracellular microorganisms. HLA variants were found to confer susceptibility or resistance to disease. In Brazilian *T. gondii*-seropositive AIDS patients, HLA-B35 antigen was associated with the susceptibility to chorioretinitis (Rodrigues *et al.* 2004). Meenken *et al.* (1995) reported an increased frequency of the HLA-Bw62 antigen in patients with severe ocular involvement. HLA genes are also indicated in determining the development of TE. For example, HLA-DQ3 gene is a genetic marker of susceptibility to TE, but HLA-DQ1 is a resistance marker among Caucasians (Suzuki *et al.* 1996). HLA-DQ3 also seems to be a genetic marker of susceptibility to hydrocephalus in infants with congenitally infection (Mack *et al.* 1999). Using a transgenic mouse model, the relationships between HLA-DQ3 and -DQ1 genes and brain susceptibility or resistance to *T. gondii* infection are confirmed (Mack *et al.* 1999). In HIV-1 infected patients who are Latin American Caucasian, the presence of HLA-DQB and DRB1 alleles could be considered as risk factors for developing neurological opportunistic infections, mainly TE (de Sorrentino *et al.* 2005).

Evidence indicates that polymorphisms in cytokine genes known to influence the course of *T. gondii* infection are important factors triggering OT occurrence. For example, several studies have reported an association between the polymorphism in IFNγ and retinochoroiditis toxoplasmosis susceptibility (Albuquerque *et al.* 2009, Peixe *et al.* 2014). Scientists in Brazil have suggested an association between patients with OT and genetic polymorphisms in several cytokine genes including IL-10, IL-1 and IL-6 (Cordeiro *et al.* 2008a, 2008b, 2013). Notably, the TNF-a polymorphism (−308G/A), a risk factor for a number of inflammatory and infectious diseases, was not associated with occurrence or recurrence of OT (Cordeiro *et al.* 2008c). Peixoto-Rangel *et al.* (2009) reported an association between polymorphism in toll-like receptor TLR9 and toxoplasmic retinochorioditis in Brazil. Recently, study found that polymorphism in the intracellular pattern-recognition receptor NOD2 was also involved in toxoplasmic retinochorioditis in Brazil (Dutra *et al.* 2013).

In large cohort of mother-child pairs from Europe and North America, Jamieson *et al.* (2008) have showed that ocular and brain disease in CT associate with polymorphisms in *ABCA4* (ATP-binding cassette transporter, subfamily A, member 4), while polymorphisms

at *COL2A1* (type II collagen) associate only with ocular disease. Recent follow-up work in severe congenital disease has noted that type II serotype is more frequent in patients bearing susceptibility alleles of COL2A and/or ABCA4 (McLeod *et al.* 2012). The presence of human genetic risk factor provides an explanation for how type II parasites cause severe disease. Moreover, polymorphisms at the purinergic receptor P2X7, a member of the NOD-like receptor family NALP1, and an endoplasmic protease ERAP1 have been shown to influence susceptibility to congenital *T. gondii* infection (Jamieson *et al.* 2010, Tan *et al.* 2010, Witola *et al.* 2011).

Rh phenotypes (rhesus-positive or rhesus-negative) are determined by the highly immunogenic RhD protein on the erythrocyte membrane. In the setting of latent toxoplasmosis, several studies have shown that RhD positivity confers protection against effects on motor performance, personality, and excessive increase of body weight in pregnancy (Flegr *et al.* 2008, Novotna *et al.* 2008, Kankova *et al.* 2010). These results indicate that genetically susceptible patients are probably less able to control *T. gondii* infection than genetically resistant patient.

Conclusions

Human infections with *T. gondii* display a wide range of clinical spectrum. This variation is likely to be a consequence of many factors including human and parasite genotypes as well as timing of infection and environmental factors such as co-infections and nutrition. From the literatures, it is clear that virulent strains are significantly more often associated with increased frequency and severity of human toxoplasmosis, but the extent to which parasite genotype directly contributes to the clinical severity is lacking. It will become clearer when information on the strains responsible for the more frequently encountered asymptomatic infections are available. Currently, there is little genotype information from asymptomatic cases and such information likely will await the improvements of serotyping methods. There is an urgent need to identify additional suitable peptides to predict the infecting strain genetically distinct from the three lineages. This information could substantially alter patient treatment, with more aggressive monitoring and treatment of infections directed at individuals with the highest risk of serious consequences from infection. Incidentally, the significance of highly virulent strains can cause severe diseases in immunocompetent patients and might implicated in brain disorders such as schizophrenia should led to reconsideration of toxoplasmosis.

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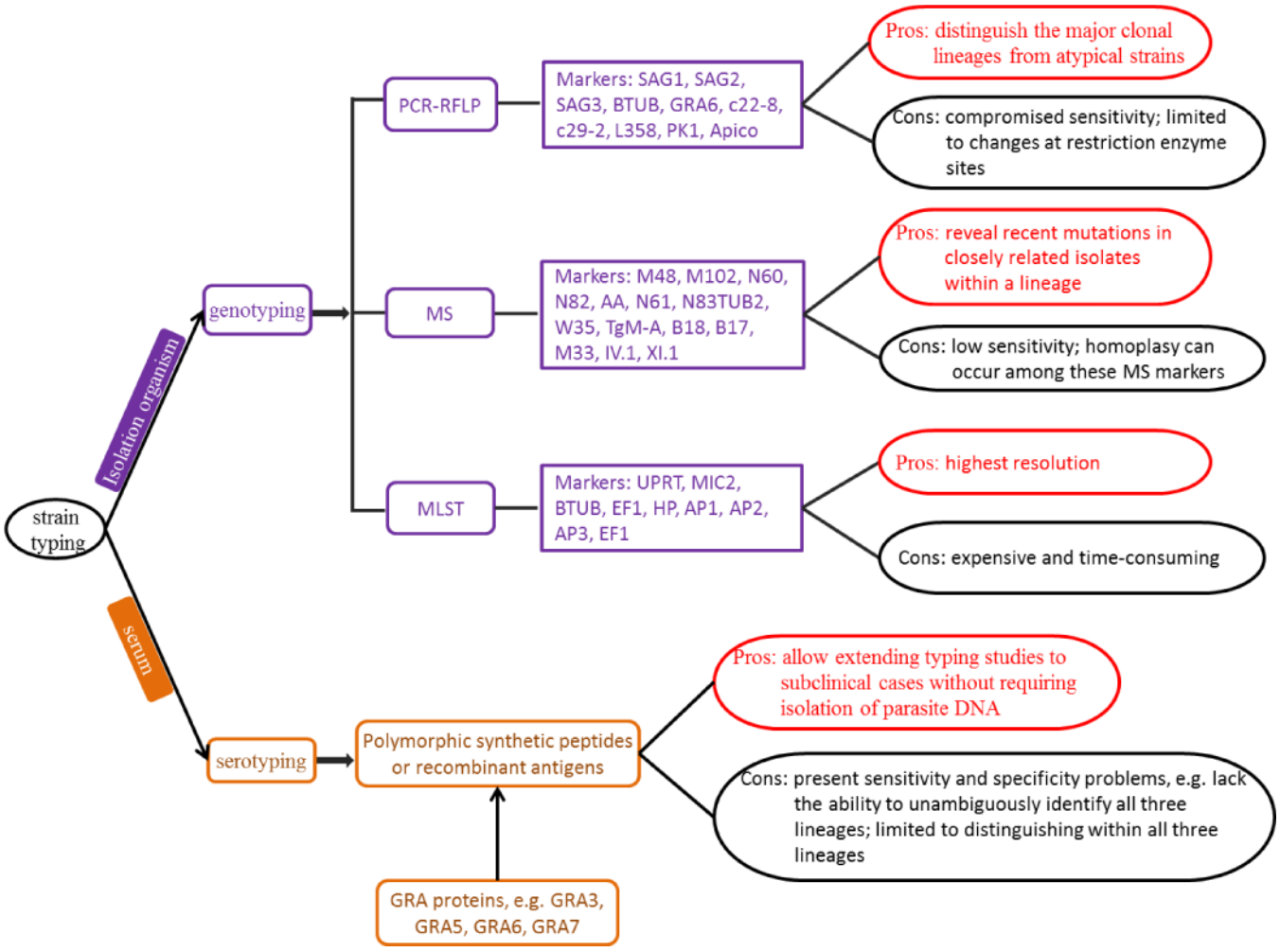

Figure 1.

An overview of the methods that commonly used for *T. gondii* strain typing and their relative advantages and limitations. PCR-RFLP, restriction fragment length polymorphism analysis; MS, microsatellite analysis; MLST, multilocus sequence typing analysis.

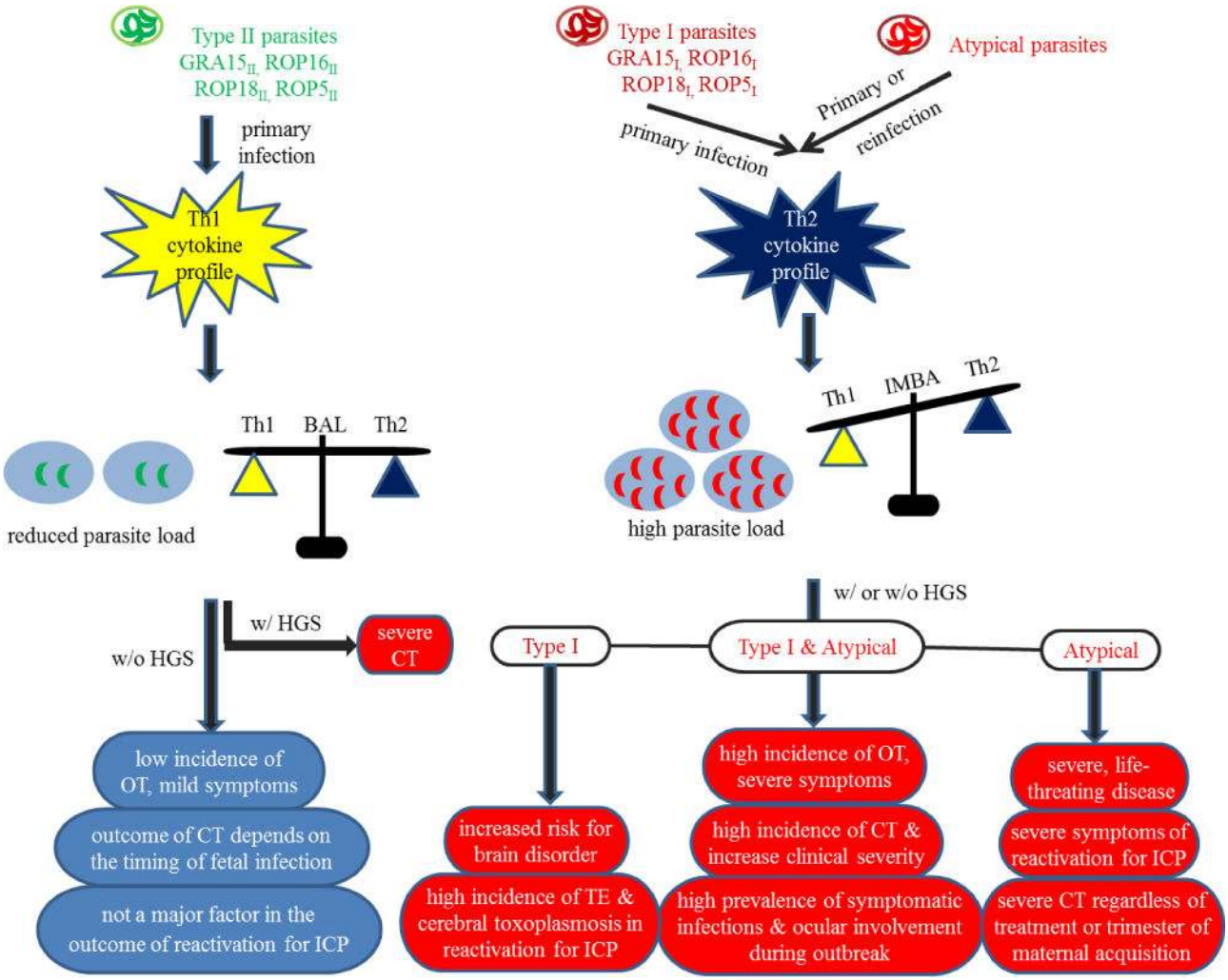

Figure 2.

An overview of the impact of *T. gondii* strains on the severity of human diseases. Several effectors of *T. gondii* (GRA15, ROP16, ROP18 and ROP5) were identified as major factors that contributed to strain-specific differences in virulence in the mouse model. The virulent isoforms of these effectors are expressed in the type I strain, whereas avirulent isoforms are secreted by type II strain. And relatively little is known regarding *T. gondii* effectors of the atypical strain. Allele types are shown in subscript (i.e. ROP_I denotes the allele in type I strains). Infection with type II strains triggers Th1 immune responses which is sufficient to control parasite burden. At later time point, a balance between the pro- and anti-inflammatory responses has been reached and the infection is then mostly asymptomatic. However, serve symptoms can occur when infection is associated with human genetic risk factors. Infection with type I or atypical strains gives rise to Th2 immune response which allows the parasite to multiply and dissemination. Finally, there is an imbalance between the pro- and anti-inflammatory responses due to a shift to a Th2 type. As a consequence, infection is likely to have symptoms in patients with or without genetic susceptibility. BAL, Balance; IMBA, imbalanced; w/, with; w/o, without; ICP, immunocompromised patient;

HGS, host genetic susceptibility; OT, ocular toxoplasmosis; CT, congenital toxoplasmosis; TE, toxoplasmic encephalitis.