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TOXOPLASMA GONDII, "NEW" GENOTYPES AND VIRULENCE

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Summary:

Toxoplasma gondii has been described as a parasite with a low genetic diversity and a clonal population structure. The three main clonal lineages designated as type I, II or III largely predominate in Europe and North America. But strains not related to these main lineages circulate, notably, in other continents. They possess a shuffled combination of alleles that typify the three clonal types and unique polymorphisms detected by multilocus analysis. The population structure of *Toxoplasma* in these continents is also characterized by a higher genetic diversity associated with a lower linkage desequilibrium suggesting a role for genetic exchange. Due to their genomic diversity, it is difficult to draw global conclusions about their virulence. However, most of them are virulent in mice at isolation. Several reports also suggest a higher pathogenicity in humans and an association with ocular toxoplasmosis or severe cases of acquired toxoplasmosis in immunocompetent patients.

KEY WORDS : *Toxoplasma gondii*, genotype, virulence.

The first genotyping studies on *Toxoplasma gondii* strains, performed on a limited number of laboratory strains and isolates mainly from France or from USA, lead to the description of a clonal population structure with three main lineages, designated as type I, II and III, related to mouse-virulence (Dardé *et al.*, 1988, 1992; Sibley & Boothroyd, 1992; Howe & Sibley, 1995). But genotypes not belonging to the three main lineages were found predominant in other continents where the population structure of *Toxoplasma* was more complex, with a higher genetic diversity than initially described. These "new" genotypes, or more exactly newly discovered genotypes, were designated, depending on the authors, as atypical, exotic, recombinant, or non archetypal genotypes. The description of these atypical genotypes offers new perspectives in the analysis of virulence determinants.

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TOXOPLASMA GENOTYPES

THE THREE MAIN CLONAL LINEAGES

The three main types respond to the criteria for a clonal population structure of *Toxoplasma* (isolation of identical multilocus genotypes over large geographic areas and at interval of several years, and a strong linkage disequilibrium) (Tibayrenc *et al.*, 1991). This simple clonal structure is accompanied by a low genetic divergence between the three main lineages (only ≈ 2 % divergence at the DNA sequence level between lineages). A large majority (84 %) of the of the single nucleotide polymorphisms (SNPs) identified among the three types are type I and II SNPs, type III polymorphisms (only 16 %) being located mainly on chromosome IV (Boyle *et al.*, 2006). The assymetrical distribution of SNPs on chromosomes indicates that 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. The limited genetic diversity within each of these lineages and the low divergence between lineages strongly suggest that these three clonal lineages have expanded as the dominant strains relatively recently, from a common ancestor 10,000 years ago (Su *et al.*, 2003).

"NEW" GENOTYPES

Extension of epidemiological screening across a wider geographical and host range and multilocus analysis of isolates revealed a more complex population structure than initially described, with higher levels of variation and recombination among some parasite populations (Ajzenberg *et al.*, 2004; Lehmann *et al.*, 2004; Su *et al.*, 2003).

Multilocus and multi-chromosome genotyping of isolates from South America, Asia and Africa with PCR-RFLP or microsatellite markers revealed that the majority of them possess type I, II or III alleles (mainly I and III), identical to those found in the three major lineages, but these have segregated differently among the loci analyzed. These isolates, presenting different mixtures of classical alleles can be considered as

recombinant genotypes. "Atypical", "unusual", "non archetypal" or "exotic" strains are also described. At some loci, these atypical strains show evidence of the allele patterns that typify the clonal lineages, but they have also many unique polymorphisms and "novel" alleles. The classification of an isolate either as a recombinant or an atypical isolate is partly artificial as it depends on the number and the discriminating power of genetic markers. The increasing number of genetic markers used for genotyping may reveal unique polymorphisms or mixtures of classical alleles in isolates previously classified as related to one of the three classical types. Despite the existence of unique polymorphisms and a higher allelic diversity, the level of global genetic polymorphism of sequences remained low (Grigg *et al.*, 2001; Ajzenberg *et al.*, 2004).

Some of these atypical genotypes are detected in multiple locations. They may correspond to new successful clonal lineages (Pena *et al.*, 2007).

THE GEOGRAPHICAL STRUCTURATION OF *TOXOPLASMA* POPULATION

EUROPE, USA

The three main types account for a large majority of isolates in North America and Europe. Among these three lineages, the type II is largely predominant in published studies. In France, it is found in more than 90 % of human congenital toxoplasmosis, but also in all isolates originating from a large variety of animals (Ajzenberg *et al.*, 2002). More studies are needed to confirm if this pattern of *Toxoplasma* population is observed throughout Europe. Preliminary studies seem to indicate that, for instance, type III or type I may be more frequent in Portugal or Spain (Fuentes *et al.*, 2001, de Sousa *et al.*, 2006). Similarly across North America, the situation might not be the same from the Eastern to the Western coast.

SOUTH AMERICA

Strains from South America are highly divergent from those of Europe or North America. The high genetic diversity observed in this continent is maximum in the Amazonian area, with many unique polymorphisms (Ajzenberg *et al.*, 2004). Although type II isolates have been found in Chile (Dubey *et al.*, 2006), it seems very rare elsewhere in South America. Genetically distinct isolates are found in different regions of South America (Dubey *et al.*, 2007c). Common clonal lineages, different from the three classical types, may circulate on this continent (Pena *et al.*, 2007).

AFRICA, ASIA

Asia and Africa are still underexplored. First reports from Asia (Dubey *et al.*, 2007a, 2007b, 2007d) reveal a more limited genetic diversity than in South America, with some genotypes common to both areas. The few strains isolated from African patients possess a fixed combination of type I and III alleles which can suggest the existence of a clonal African type (Ajzenberg *et al.*, 2004) possibly related to one of the Brazilian types (Pena *et al.*, 2007).

HIGHER GENETIC DIVERSITY IN WILDLIFE

Frequent genetic exchange, generating a variety of recombinants and a higher genetic diversity, may be more common in *Toxoplasma* isolates in the wildlife cycle (e.g. French Guiana), or in areas where breeding is recent or not intensive and cat domestication recently introduced (e.g. Brazil, West or Central Africa, the Caribbean). A few recombinant isolates have also been described from wildlife in North America (bears and deer) (Howe & Sibley, 1995; Ajzenberg *et al.*, 2004). In Europe or North America, intensive breeding of a narrow range of domestic meat producing animals together with cat domestication offered a major niche to the three main lineages and leaded to an impoverishment of genetic diversity (Lehmann *et al.*, 2003; Ajzenberg *et al.*, 2004).

TOXOPLASMA GENOTYPES AND EXPERIMENTAL VIRULENCE

VIRULENCE OF THE THREE MAIN CLONAL LINEAGES

Virulence is usually defined in the mouse model after intraperitoneal inoculation of given numbers of tachyzoites: type I isolates are highly virulent leading to death of mice less than 10 days after inoculation of < 10 tachyzoites (LD100 ≈ 1); avirulent strains (type II or III strains) allow survival after inoculation of > 10^3 tachyzoites (LD50 > 10^3). In mouse, the high virulence is partially due to the triggering of an uncontrolled TH1 type cytokines response which contributes to tissue pathology (Gavrilescu & Denkers, 2001; Nguyen *et al.*, 2003). *In vitro*, the mouse-virulent strains display enhanced migration under soft agarose plates, enhanced transmigration across polarized epithelia or across extracellular matrix, and a higher rate of *ex vivo* penetration of lamina propria and submucosa (Barragan & Sibley, 2002). Virulence has also been linked to a higher rate of interconversion from tachyzoite to bradyzoite in cell culture. The ability to cross epithelial barriers rapidly and reach the bloodstream within hours post-infection might be an impor-

tant predeterminant of parasite dissemination in mice or other susceptible host species. A higher growth rate in cell culture either due to a higher reinvasion rate or to a shorter doubling time (Saeij *et al.*, 2005) may also explain the higher tissue burden observed in mice infected with virulent strains.

Although these *in vitro* studies demonstrate different intrinsic properties of the different strains, the expression of this virulence in a given host species is a more complex trait which depends on several host and parasite characteristics. The different host species are more or less susceptible. For example, type I strains, which are highly virulent in mice, are not pathogenic in rats which developed a subclinical chronic infection. Some rat strains, such as the Lewis rat, are even refractory to infection with a mouse-highly virulent strain (Sergent *et al.*, 2005). The genetic background in a given species, as demonstrated in different mouse strains, may also influence the expression of virulence.

Virulence of non clonal lineages

The practical implication of a clonal population structure is that biological characteristics can be attributed to a genetically well defined subset of the parasitic population (Tibayrenc *et al.*, 1991). That is the case for mouse-virulence of the three main types of *Toxoplasma*. For atypical and naturally recombinant strains, due to their different genetic background, the association between genotype and phenotype can not be predicted. They ranged from highly virulent to intermediate or non-virulent phenotype according to the differences in the combination of genes they have inherited (Grigg & Suzuki, 2003). Pena *et al.* (2007) define their virulence at isolation (*i.e.* without knowledge of infecting dose) on mortality of mice within four weeks of infection and categorize isolates into three groups: virulent (death of 100 % of mice within four weeks), intermediate virulent (30 % to less than 100 % mortality), and non-virulent (< 30 % death within four weeks). Actually, most of them exhibit a dose-dependant virulence. To detect dose-dependant variations in virulence observed with these non clonal isolates, the classical definition of experimental mouse-virulence was refined. Saeij *et al.* (2006) defined the avirulence phenotype as no mortality at any dose, whereas a "low-dose survivability" phenotype was defined by survival time after injection of 100 parasites.

The usually higher virulence of naturally recombinant strains is consistent with results of experimental crosses. A cross between two avirulent strains (type II × type III) gave rise either to avirulent progeny like their parents or to progeny with enhanced virulence (Grigg *et al.*, 2001b). Remarkable differences in the dissemination patterns in mice were observed between a virulent progenitor from this type II × III cross and its non-virulent siblings strain (Saeij *et al.*, 2005). Another experimental cross between a highly virulent type I strain and a less virulent type III strain is particularly significant as the majority of naturally recombinant strains isolated today present a mixture of type I and III alleles. It shows that mortality caused by the progeny clones of this type I × III cross ranged from low levels to 100 %, consistent with a multilocus trait (Taylor *et al.*, 2006).

Genetic background of virulence

Experimental crosses between strains with different virulence proved useful in identifying genes that determine virulence (Saeij *et al.*, 2006; Taylor *et al.*, 2006). Those genes were located mainly on chromosome VIIa and the best candidate gene encodes ROP18, a rhoptry protein of the ROP2 family, a highly polymorphic serine-threonine kinase secreted during parasite invasion. But virulence is probably not governed by one single gene. On chromosome VIIb is located the gene encoding for ROP16, an other rhoptry protein kinase which is injected into the host cell cytosol and is involved in the strain differences in induction of interleukin 12 secretion by mouse macrophages (Saeij *et al.*, 2006). Other candidate genes has been found on chromosome XII (ROP5, SAG3, Adenosine kinase) (Saeij *et al.*, 2006), and various parasitic secretions from micronemes or dense granules also play additional roles in expression of virulence (review Lebrun *et al.*, 2007)

It would be interesting to look for the polymorphism and the expression level of the virulence genes detected by these experimental crosses in naturally recombinant strains to better predict the virulence of a given isolate. A first study detected significant differences in virulence among atypical isolates according to alleles I, II and III of CS3 locus, one of the candidate virulence gene located on chromosome VIIa in close proximity to ROP18 locus (Pena *et al.*, 2007).

Genotypes and expression of *Toxoplasma* virulence in humans

Parasitic factors determining pathogenicity in humans are poorly understood. The studies have been hampered by the genetic heterogeneity of human populations and differences in parasitic factors (infectious stage, inoculum). The immune status of the host also plays a major role for an opportunistic parasite such as *Toxoplasma*.

- Congenital toxoplasmosis

In South America, the few reports about isolates from congenital cases indicates the role of type I, atypical or recombinant I/III strains in severe cases (Ferreira *et al.*, 2006; Gallego *et al.*, 2006). In the absence of systematic isolation, it is impossible to know if these strains are not also observed in asymptomatic cases. In France

where a systematic diagnosis of congenital toxoplasmosis is performed, more than 80 % are due to type II (Ajzenberg *et al.*, 2002b). Type II isolates were found in all the different aspects of congenital disease from lethal infection to latent toxoplasmosis. The main factor determining the severity of congenital infection due to type II remains the stage of pregnancy at which it is acquired, the disease being more severe after early maternal infection. But the few atypical isolates detected in this country were observed only in severe cases of congenital toxoplasmosis after late maternal infection, suggesting a higher rate of dissemination and tissue burden in the infected foetus.

• Immunocompromised patients

Among strains isolated from cases of toxoplasmosis in 85 immunocompromised patients diagnosed in France, type II predominates but it was noted a larger proportion of recombinant I/III isolates, compared to congenital toxoplasmosis. But all these recombinant I/III isolates were found in African patients who reactivated a chronic infection probably acquired in Africa (Ajzenberg *et al.*, submitted), showing the main role of geographical origin in the type of the strain infecting these patients. There was no significant difference in the clinical manifestations and the outcome according to the genotype. In USA, Khan *et al.* (2005) found a majority of type I strains or strains containing type I alleles in eight AIDS patients. They concluded that these strains were more prone to reactivate, but the limited number of samples tested and the lack of information on the geographical origin of the patients might bias this conclusion.

• Immunocompetent patients

The higher virulence of some atypical genotypes is more apparent in immunocompetent patients. In acquired ocular toxoplasmosis, an unusual abundance of type I or recombinant genotypes I/III have been found in USA (Grigg *et al.*, 2001c). The high occurrence of ocular toxoplasmosis in Brazil has been attributed to atypical or recombinant genotypes circulating in this country (Khan *et al.*, 2006). This may also explain the high frequency (20 % of 97 cases) of ocular involvement in the Victoria outbreak, in British Columbia where an atypical cougar isolate was suspected (Burnett *et al.*, 1998), and the 100-fold higher incidence of ocular toxoplasmosis in patients born in Africa compared to patients born in Britain (Gilbert *et al.*, 1999). The virulence of atypical strains is also obvious in the cases of severe toxoplasmosis observed in immunocompetent patients with multi-organ failure (Carme *et al.*, 2002). Several deaths due to toxoplasmosis were observed in these immunocompetent patients. These infections, all due to atypical strains, were acquired after wild game consumption or after drinking water in the wild forest of French Guiana. Occasional reports of such severe cases due to atypical strains were observed in other countries (De Salvador-Guillouet *et al.*, 2005). Although these reports strongly support the idea of a higher virulence of some atypical strains in humans, the role of the inoculum and of the host genetic background should not be forgotten. Interestingly, in an outbreak due to a single atypical strain (Demar *et al.,* 2007), some patients died or were seriously ill whereas other patients presented only with mild symptoms.

CONCLUSION

Relationships between genotype and human disease certainly exist, but are still difficult to assess due to the role of host immune status and genetic background on the control of infection, and of other parasitic factors such as the infecting dose or parasite stage. The situation for the "new" genotypes is even more complex, due to their diversity and to the combination of genes. Recombination may lead to strains that acquire new pathogenic mechanisms, as suggested in humans by the severity of cases of toxoplasmosis in immunocompetent patients, or in mice by the higher virulence of the progeny of experimental crosses. The new genotypes resulting from these recombinations can expand in the population leading to emergent diseases.

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