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Genotypic Characterization of *Toxoplasma gondii* Strains Associated with Human Toxoplasmosis in Spain: Direct Analysis from Clinical Samples

ISABEL FUENTES,* JOSE M. RUBIO, CARMEN RAMÍREZ, AND JORGE ALVAR

Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

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Genetic analysis of the *SAG2* locus was performed to determine the prevalence of the different genotypes of *Toxoplasma gondii* (strain types I, II, and III) associated with human toxoplasmosis in Spain. This determination was made directly from primary clinical samples, obviating the previous process of isolation in mice or cell culture. A total of 34 isolates of *T. gondii*, collected from immunocompromised patients and congenital infection cases, were analyzed. Restriction fragment length polymorphism in PCR-amplified *SAG2* products was used to group strains into one of the three genotypes of *T. gondii*. Complete characterization of the *SAG2* gene was successful in 76.5% of the cases, demonstrating the feasibility of direct genotype analysis from clinical samples of different origins. Strains of *T. gondii* type II were the most prevalent in immunocompromised patients, with 52% of cases, while strains of type I were present in 75% of the congenital infection cases. These data differ from previous reports that show type II strains to be mostly associated with all kinds of human toxoplasmosis. These differences might be an effect of selection in the process of culture and isolation of the samples performed by other researchers prior to strain characterization.

The protozoan *Toxoplasma gondii* is an obligate intracellular parasite that infects humans and a broad spectrum of vertebrate hosts. The transmission of *T. gondii* occurs by ingestion of oocysts shed from feline feces, by ingestion of *T. gondii* cysts from chronically infected tissues, or by vertical transmission (23).

Between 15 and 85% of the world adult human population is chronically infected with *T. gondii* depending on geographical location (4).

Toxoplasmosis has variable outcomes in the host. Immunocompetent infected individuals show mild symptoms or may remain asymptomatic, while infection in congenitally infected children and in immunocompromised persons (AIDS patients, organ transplant recipients, and cancer patients) causes high rates of morbidity and mortality (15). The progression and severity of the disease differ in patients due to several variables, including host and parasite genetics (8, 21). It is well-known that the virulence of *T. gondii* differs in animals, depending on the *T. gondii* strain (16). The identification of a possible correlation between the severity or type of disease and strain genotyping might be very important for determination of the correct treatment and the possible outcome of the disease in each human case (21).

Genetic analysis of strains indicates that the propagation of *T. gondii* is primarily by clonal, asexual, or uniparental sexual reproduction, while sexual recombination between different strains of the parasite is exceptional in natural populations (20).

* Corresponding author. Mailing address: Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Cra. Majadahonda-Pozuelo Km 2, Majadahonda, 28220 Madrid, Spain. Phone: 34-915097901, ext. 3627. Fax: 34-915097034. E-mail: ifuentes@isciii.es.

T. gondii strains have been subdivided in two or three major groupings using different methods of characterization, such as isoenzyme electrophoresis, restriction fragment length polymorphism (RFLP), PCR, or random amplified polymorphism DNA (2, 3, 9, 12, 20). Howe and Sibley (14), analyzing six independent single-copy loci by PCR-RFLPs, described three clonal lineages, named types I, II, and III, which correspond to the genetic analysis of the polymorphic surface antigen 2 locus (*SAG2*) (12). Several reports analyzing samples isolated in mice or in vitro cultures show that type II is the most prevalent genotype (3, 12, 14, 18–20).

The genetic analysis of *T. gondii* based on amplification of the *SAG2* locus requires small amounts of DNA, thus allowing it to be used directly on clinical samples. The aim of this work was to determine the lineage types of *T. gondii* associated with human toxoplasmosis in Spain. For this purpose, genetic analysis of the *SAG2* locus for amplification of the DNA obtained directly from clinical samples was performed, obviating the previous process of isolation in mice or cell culture.

MATERIALS AND METHODS

Clinical samples. Cerebrospinal fluid (CSF), blood, aqueous humor, and lung and brain biopsy samples were obtained from immunocompromised patients with toxoplasmosis, along with amniotic fluid, blood, and urine samples from pregnant women and abortion ascitic fluid and blood samples from newborn babies with congenital infections. A total of 34 samples from 33 patients, 20 immunocompromised patients and 13 infants with congenital infections, with different pathologies were included in the study (see Table 1).

Samples of CSF (0.5 ml) and urine and amniotic fluid (2 to 5 ml) were concentrated by centrifugation at 1,800 × *g* for 10 min, and the pellets were stored at −20°C. The rest of the samples were stored at 4°C, until analyzed.

Experimental samples. The following representative strain types of *T. gondii* were used for standardization and as controls of PCR assays: strain RH (type I), strains Beverly and Me49 (type II), and strain C56 (type III) (14). Parasites were grown and maintained by bioassay in mice, and samples of chronically infected mouse brain were used as positive controls in PCR assays and genotype analysis.

Isolation of DNA. DNA extraction from blood and paraffin-embedded tissues was performed using Wizard and Dexat genomic DNA purification kits (Promega, Madison, Wis.) in accordance with the manufacturer's instructions. The rest of the samples were incubated in 100-μl portions of lysis buffer (10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg of gelatin per ml, 0.5% Tween 20, 20 μg of proteinase K) at 55°C, with shaking for 90 min. After inactivating the proteinase K at 94°C for 10 min, the suspension was centrifuged at 12,000 rpm for 5 min, and the supernatant, which contained the DNA, was moved to a new tube.

Detection of *T. gondii* by PCR. *T. gondii* infections were initially confirmed by nested PCR amplification of the repetitive and conserved gene B1 (1, 6).

Genotype analysis. The lineage type was determined by restriction fragment of the amplified *SAG2* gene of *T. gondii* using two nested PCRs separately amplifying the 5′ and 3′ ends of the gene (12).

The PCR mix for the nested reactions consisted of 75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.001% bovine serum albumin, 200 μM each of the four deoxynucleoside triphosphates, 0.5 pmol of each primer, 1 U of DNA polymerase (Biotools, Madrid, Spain), and, as a template, 1 to 20 μl of extracted sample DNA for the first reactions and 5 μl of a 1:100 dilution of the products of the first amplifications for the second reactions, in a final volume of 50 μl.

All the PCRs were performed in a 2400 GeneAmp PCR system thermal cycler (Perkin-Elmer, Norwalk, Conn.). The first step of amplification was 5 min of denaturation at 94°C. This step was followed by 40 cycles, with 1 cycle consisting of 45 s at 94°C, 45 s at the annealing temperature for each pair of primers, and 60 s at 72°C. The final cycle was followed by an extension step of 10 min at 72°C.

The 5′ end was amplified using primers SAG2F4 (5′-GACCTCGAACAGG AACAC-3′) and SAG2R4 (5′-GCATCAACAGTCTTCGTTGC-3′) in the first amplification, at an annealing temperature of 60°C. In the second reaction, the internal primers SAG2F (5′-GAAATGTTTCAGGTTGCTGC-3′) and SAG2R2 (5′-GCAAGAGCGAACTTGAACAC-3′) were used, with 58°C as the annealing temperature.

Amplification of the 3′ end was performed with primers SAG2F3 (5′-TCTG TTCTCCGAAGTGACTCC-3′) and SAG2R3 (5′-TCAAAGCGTGCATTATC GC-3′) for the first amplification at an annealing temperature of 58°C and with the internal primers SAG2F2 (5′-ATTCTCATGCCTCCGCTTC-3′) and SAG2R (5′-AACGTTTCACGAAGGCACAC-3′) for the second amplification at an annealing temperature of 55°C. The 5′-end amplification is expected to yield a product of 241 bp, while the 3′-end amplification must give a product of 221 bp in both cases for any strains of *T. gondii*.

In order to avoid possible contamination, several measures, such as separate space to set up PCRs, filter tips, etc., were taken, as well as different negative controls (no DNA, uninfected blood, and extracted no DNA), and positive controls from different strains of *T. gondii* were used in order to locate any possible contamination.

The amplified products were purified with Bioclean Purification Columns (Biotools) and digested with *Sau*3AI (5′-end products) and with *Hha*I (3′-end products). The PCR products and the restriction fragments were analyzed by 2% agarose gel electrophoresis.

Restriction digestion of 5′-end-amplified products with *Sau*3AI distinguished the type III strain from type I and II strains and digestion of the 3′-end-amplified fragments with *Hha*I differentiated type I and III strains. Controls for lineage types were prepared from RH (type I), Beverly (type II), Me49 (type II), and C56 (type III) strains of *T. gondii*.

In those cases in which some of the samples were seen to be inhibited in the nested PCR, the rest of the reaction mix of the first amplification process was recovered and cleaned using the Bioclean Purification Columns (Biotools), in accordance with the manufacturer's protocol, and 5 μl of the elute was used as a template in the nested PCR.

The method used for the characterization of *T. gondii* strains implied digestion with restriction enzymes of the fragments amplified. The acquisition of digested and nondigested products from the same purification set means that the products obtained are not due to any contamination in the purification or amplification process.

RESULTS

A collection of 34 samples from 33 patients (15 immunosuppressed human immunodeficiency virus [HIV]-positive patients, 5 immunosuppressed HIV-negative patients, 5 pregnant women, and 8 newborn babies) were analyzed in order to characterize the genotype of *T. gondii* present in each sample (Table 1). The isolation of DNA was carried out directly from the samples without previous isolation in culture or mice. Genetic analysis was performed by PCR-RFLP at the *SAG2* loci of *T. gondii*.

Previously, all the samples were confirmed for the presence of the parasite by nested PCR of the B1 *T. gondii* gene. In all the samples, as well as in the four reference strains, the nested PCR rendered the expected fragment of 97 bp, while all the negative controls, including uninfected samples of mouse brain and human blood, were negative.

Amplification of both ends of the *SAG2* gene was successful in the four reference strains and in 26 of 34 clinical samples (76.5%). In another six cases (18%), only the 3′ end of the *SAG2* gene was amplified, and in the other two (6%), only the 5′ end was amplified (Table 1). Nine of these fragments were obtained by purification and new nested amplification of previously inhibited PCR. In another nine cases, this strategy did not work and the method was impossible to repeat, as new samples were not available.

The patterns of digestion of the four reference strains corresponded to the expected results. The RH strain was characterized as genotype I because the 3′- and 5′-end-amplified fragments of the *SAG2* gene were undigested with the corresponding restriction enzymes. The Beverly and Me49 strains were characterized as genotype II since the 3′-end fragments of the *SAG2* gene were digested with *Hha*I while the 5′-end fragments were undigested. The C56 strain was characterized as genotype III because the 3′-end fragments of the *SAG2* gene were undigested while the 5′-end fragments were digested by *Sau*3AI.

Figure 1 shows the digestion patterns of the various strains studied.

Genotyping of the 25 fully studied samples rendered the following results: 10 of type I (40%), 10 of type II (40%), and 5 of type III (20%). The eight strains which were partially characterized resulted in two of type I or II (non-type III) and six of type I or III (non-type II). The two brain biopsy samples from the same patient (patient 14 IHIV+) rendered the same result, type III (Tables 1 and 2).

Of the samples from the 17 immunosuppressed patients that were fully characterized, four (23.5%) were type I, nine (53%) were type II, and the other four (23.5%) were type III. The other three immunosuppressed patients were infected in two cases by non-type III strains of *T. gondii*, and the third was infected by a non-type II strain. On the other hand, of the eight samples from congenital toxoplasmosis cases that were fully characterized, six (75%) were type I, while one (12.5%) was type II and one (12.5%) was type III. The other five samples from newborn babies for which total characterization was not possible were infected by non-type II strains (Tables 1 and 2).

In general, there is no clear correlation between strain genotype and symptomatology. In immunocompromised patients, the three genotypes produce symptomatology related to neurological diseases, associated in several cases with pneumonia and other related lung diseases; in two cases, with strain type II, the patients show eye diseases without other associated symptoms. In the cases of congenital toxoplasmosis in which it was possible to follow up on the patients, there was not a clear association between disease and the genotype of *T. gondii*. The two newborn patients that presented strain types II and III and

TABLE 1. Patients, clinical symptoms, origin of samples and genotype of different *T. gondii* strains characterized in this study

Patient group and designation[a]	Clinical symptoms[b]	Sample	Nested PCR RFLP result[c]		
			SAG2-3′	*SAG2-5′*	Type
Immunocompromised					
1 IHIV+	NS	CSF	−	−	I
2 IHIV+	NS	CSF	−	−	I
3 IHIV+	GS, cachexia	Blood	−	−	I
4 IHIV−	No data	Blood	−	−	I
5 IHIV+	NS	CSF	+	−	II
6 IHIV+	NS	CSF	+	−	II
7 IHIV+	NS	CSF	+	NA	II
8 IHIV+	NS	Brain (from biopsy)	+	−	II
9 IHIV+	NS	CSF	+	−	II
10 IHIV−	NS	Brain (from biopsy)	+	−	II
11 IHIV−	PS, fever	Lung (from biopsy)	+	−	II
12 IHIV+	Eye disease	Vitreous humor	+	−	II
13 IHIV−	Eye disease	Vitreous humor	+	−	II
14 IHIV+	NS, PS, retinochoroiditis	CSF	−	+	III
15 IHIV+	NS	2 brain samples (from biopsies)	−	+	III
16 IHIV+	NS	Blood	−	+	III
17 IHIV+	NS	CSF	−	+	III
18 IHIV+	NS	CSF	NA	−	non-III
19 IHIV+	NS	CSF	NA	−	non-III
20 IHIV−	NS	CSF	−	NA	non-II
Congenital infection					
1 Cmaternal	No data on newborn	Amniotic fluid	−	−	I
2 Cmaternal	No data on newborn	Amniotic fluid	−	−	I
3 Cmaternal	No data on newborn	Amniotic fluid	−	−	I
4 Cmaternal	No data on newborn	Maternal urine	−	−	I
5 Cnewborn	CC, VD, chorioretinitis	Blood	−	−	I
6 Cnewborn	Abortion, cardiopathy	Abortion ascitic fluid	−	−	I
7 Cnewborn	Newborn asymptomatic	Blood	+	−	II
8 Cnewborn	Newborn asymptomatic	Blood	−	+	III
9 Cnewborn	No data on newborn	Blood	−	NA	non-II
10 Cnewborn	No data on newborn	Blood	−	NA	non-II
11 Cnewborn	Newborn asymptomatic	Blood	−	NA	non-II
12 Cnewborn	Newborn asymptomatic	Blood	−	NA	non-II
13 Cmaternal	No data on newborn	Maternal blood	−	NA	non-II

[a] The patient designations indicate the HIV status and time of diagnosis of congenital infection as follows: IHIV+, immunocompromised HIV-positive patient; IHIV−, immunocompromised HIV-negative patient; Cmaternal, maternal diagnosis of congenital infection; Cnewborn, newborn diagnosis of congenital infection.
[b] Abbreviations; NS, neurological symptoms; GS, general symptoms; PS, pulmonary symptons; CC, cerebral calcifications; VD, ventricle dilation.
[c] *SAG2*-3′ and *SAG2*-5′, 3′- and 5′-end-amplified fragments of *SAG2*; +, product amplified digested; −, product amplified not digested; NA, product not amplified; non-III, non-type III; non-II, non-type II.

the three non-type II cases that were not fully characterized were asymptomatic, while of the two patients with genotype I, one was aborted and the other was seriously ill.

DISCUSSION

The main objective of this report has been to show the possibility of characterizing the different *T. gondii* genotypes directly from clinical samples, since the acquisition, isolation, and maintenance of strains are difficult and require a long period of time, besides preventing artificial prevalence of strains associated with enrichment of parasite prior to diagnosis in the culture process.

Fifty-nine of 68 (86.8%) PCR fragments (3′ end and 5′ end amplified of the 34 clinical samples) were amplified directly from the clinical samples. Nine of these fragments were obtained after cleaning the first PCR mix and repeating the nested PCR; in another nine cases, this strategy did not work, and total characterization of eight strains was not possible. Total characterization of the *SAG2* gene was successful in 26 of 34 clinical samples (76.5%). These results show that it is possible to use direct characterization not only to save time but also, and most importantly, because at least partial characterization was possible with all the samples. When using indirect characterization by previous growth of the parasite in cell culture or in mice, only 40% of the samples are able to grow (11). Moreover, the use of direct characterization also avoids misleading results due to artificial selection in relation to the culture process (5). This direct process of genotyping allows material kept frozen or in other adverse culture conditions to be characterized.

The frequencies of the genotypes of the strains fully studied were 40% of type I, 40% of type II, and 20% of type III (Table 2). In other reports, the type II genotype is the most prevalent in humans with toxoplasmosis, with values of between 70 and 81% (3, 7, 12, 14, 20), but our data suggest that these differences are not so high, especially when it is considered that at least 64% of the individuals in our study, including these individuals without total genotyping characterization, carried non-type II strains.

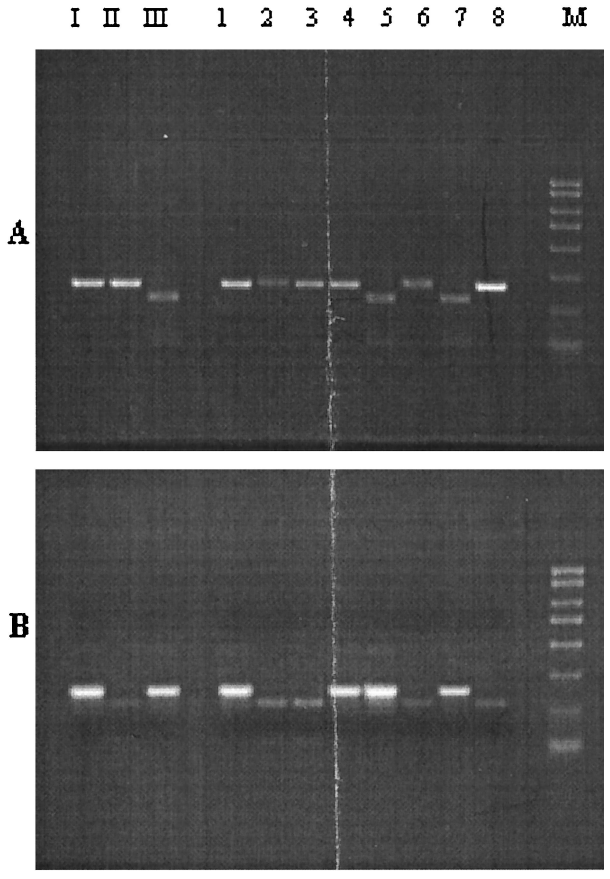

FIG. 1. Agarose gel electrophoresis analysis of *SAG2* PCR amplification products and restriction digests from *T. gondii*-infected clinical samples. (A) *Sau*3AI restriction analysis of the 5′ amplification products and (B) *Hha*I restriction analysis of the 3′ amplification products. Lane I, DNA from strain RH (type I); lane II, DNA from strain Beverly (type II); lane III, DNA from strain C56 (type III). Lanes 1 to 8 contain strains from clinical samples as follows: lane 1, CSF from patient 1 IHIV+ (type I); lane 2, CSF from patient 5 IHIV+ (type II); lane 3, blood from patient 7 Cnewborn (type II); lane 4, blood from patient 5 Cnewborn (type I); lane 5, CSF from patient 14 IHIV+ (type III); lane 6, vitreous humor from patient 12 IHIV+ (type II); lane 7, blood from patient 8 Cnewborn (type III); lane 8, brain (from biopsy) from patient 8 IHIV+ (type II). Lane M, molecular weight markers.

There are other differences observed, depending on the risk group infected; while Howe et al. (12) reported that the 13 cases of congenital toxoplasmosis studied were all type II, we found in the same number of congenital infection cases that only 8% were type II, while 46% were type I, a further 8% were type III, and 38% were non-type II. In the immunocompromised patients, we found a maximum of 55% of infection by *T. gondii* type II, including two individuals characterized as non-type III, in contrast to the 76% found by Howe et al. (12).

There are two possible explanations for these differences. The epidemiological prevalence and the route of transmission may be very different in Spain and France, where most studies were performed. The use of cell culture or mice to isolate and grow the parasites from the clinical samples, as has been the case in previous reports, might produce sensitive variations in the observed genotyping frequencies due to an effect of differential selection of the strains.

In general, there is no clear correlation between strain genotype and symptomatology. In AIDS and other immunocompromised patients, the three genotypes produce symptomatology related to neurological diseases. The low level of gamma interferon and other factors related to the immune system in these patients might increase the possibility of reactivation of the infective forms of the parasite, especially of type II, due to the high potential to develop bradyzoite stages, increasing the formation of cysts in the brain (8), as shown in other studies carried out in experimental immunocompromised mice where genotype II increased the number of cysts formed in the brain compared to type I (10, 21).

The number of congenital cases studied does not allow any clear correlation between symptoms and genotype to be observed, although in the cases where follow-up was possible the newborn patients that presented strain types II and III and the three cases with non-type II strains that were not fully characterized were asymptomatic, while patients with genotype I were seriously ill or were aborted. The type I genotype is considered to be the most virulent type, with a high level of parasitemia (14, 17). This might imply an increase in the risk of transplacental transmission, producing serious symptoms in the fetus or newborn. Thus, if this correlation is true, the three congenital asymptomatic cases with genotyping non-type II might be included in type III, a less virulent genotype; however, a larger number of studied cases would be necessary to prove this correlation hypothesis.

These results demonstrate that molecular epidemiological studies on *T. gondii* may be performed directly from infected-tissue samples. The nested genotyping PCR used here is a fast and highly sensitive method and may be used directly on clinical samples, avoiding the time-consuming techniques required to grow the parasite and avoiding the possible loss of samples or strain selection during culture. These studies are important, as they will provide a better understanding of epidemiology and association between parasite genotypes and human toxoplasmosis, if studies with a larger number of samples confirm the results obtained, especially in cases of congenital toxoplasmosis infection where treatment might be improved.

TABLE 2. Strain types of *T. gondii* found in human toxoplasmosis cases in Spain

Patient group	No. (%) of strain type				
	Type I	Type II	Type III	Non-type III	Non-type II
Immunocompromised	4 (24)	9 (52)	4 (24)	2	1
Congenital infection	6 (75)	1 (12.5)	1 (12.5)		5
Total	10 (40)	10 (40)	5 (20)	2	6

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