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Determination of Genotypes of *Toxoplasma gondii* Strains Isolated from Patients with Toxoplasmosis

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To determine the genotypes of *Toxoplasma gondii* strains associated with human toxoplasmosis, we developed a sensitive approach for typing parasites grown from clinical samples by short-term in vitro culture. A newly described nested PCR assay was capable of amplifying genomic DNA from as few as five parasites in the presence of host tissues. Typing was based on DNA polymorphisms at the *SAG2* locus, encoding tachyzoite surface antigen p22. Restriction fragment length polymorphisms in PCR-amplified *SAG2* products were used to classify strains into one of the three major lineages of *T. gondii*. This approach was successfully used to determine the genotypes of 68 of 72 samples that had been previously isolated from patients with congenital, cerebral, and disseminated toxoplasmosis. Type II strains of *T. gondii* were found in a majority of the samples, accounting for 55 (81%) of the 68 toxoplasmosis cases. In contrast, type I and III strains were found in only 7 (10%) and 6 (9%) of the 68 cases, respectively. The results of this study support the previous finding that type II strains are most often associated with human toxoplasmosis. Nested PCR analysis at the *SAG2* locus provides rapid assignment of *T. gondii* to a specific genotype that should be useful in analyzing a variety of clinical samples.

Toxoplasma gondii is a ubiquitous intracellular protozoan parasite that chronically infects approximately one-third of the adult human population in the United States and up to 85% of the adults in parts of Europe (3). Although infections with this parasite are typically nonpathogenic in healthy individuals, *T. gondii* causes substantial morbidity and mortality in immunocompromised patients (mainly AIDS patients and bone marrow and organ transplant recipients) (9, 10) and in congenitally infected infants (19). Substantial variation in the progression and severity of disease is observed between cases of toxoplasmosis, both congenital and immunocompromise related, and these differences are presumably due to several variables, including host (11, 17) and parasite (7, 15) genetics.

T. gondii has a highly clonal population structure (1, 6, 15), despite the opportunity for genetic recombination in the feline definitive host (4, 13). Population genetic analysis based on restriction fragment length polymorphisms (RFLPs) indicates that *T. gondii* consists of only three clonal lineages, designated types I, II, and III, which occur in both animals and humans (6). In a previous study of 68 independent human strains, more than 70% of human disease cases of toxoplasmosis were associated with the type II strains (6). A similar pattern was also seen in a largely independent group of samples analyzed by multilocus isoenzyme electrophoresis (1). In contrast, both type II and III strains were equally prevalent in a sample of 34 naturally infected animals, suggesting a specific association of parasite genotype II with clinical disease in humans (6). However, all of these previously analyzed strains were isolated by repeated passage in mice and in vitro culture, and therefore the apparent frequencies of specific lineages may have been biased against strains that were more difficult to isolate. Consequently, it would be advantageous to develop methods to directly analyze the parasite genotype from primary clinical samples or following minimal parasite growth. Direct analysis would also provide valuable information on the genotypes of *T. gondii* strains in archived samples (e.g., cerebrospinal fluid [CSF], blood, culture slides, and formalin-fixed and paraffin-embedded tissues) from patients with clinical toxoplasmosis when isolation of live strains is not feasible.

In this report, we describe the development of an amplification-based assay for genetic analysis of the *SAG2* locus. This assay was used to analyze 72 primary *T. gondii* isolates from fixed culture slides collected from toxoplasmosis patients between 1988 and 1996 at the Hôpital Saint-Louis, Paris, France.

MATERIALS AND METHODS

Experimental samples. The following representative strain types were used for standardization of PCR assays: strain RH (type I), strain Me49 or PLK (type II), and strain CEP (type III) (6). Parasites were grown in human foreskin fibroblast (HFF) cells and prepared as previously described (6). To test the sensitivity of nested *SAG2* PCR, purified RH strain tachyzoites were used to spike samples of HFF cells, CSF, or normal mouse brain tissue. HFF monolayers grown on 16-mm round coverslips were fixed in cold acetone, air dried, and spiked with freshly isolated RH strain tachyzoites prior to extraction. Human CSF was obtained from a human immunodeficiency virus-positive patient with negative toxoplasma serology; PCR amplifications of the *B1* locus were repeatedly negative for this sample. Mouse brain tissue was isolated from formalin-fixed, paraffin-embedded blocks by treatment with xylene followed by washing with 100% ethanol. Spiked and negative control samples were processed in parallel for nested *SAG2* PCR as described above. Among each set of samples analyzed, a water blank was included in all steps of the PCR to ensure the absence of contamination of samples during analysis.

Clinical samples. Clinical samples were inoculated onto MRC5 monolayers grown on glass coverslips. After 4 days of incubation, the cultures were fixed with cold acetone, dried, and incubated with a rabbit anti-*T. gondii* primary antibody, followed by a fluorescent anti-rabbit secondary antibody (2). The coverslips were counterstained with Evans blue, mounted in phosphate-buffered saline–glycerol, and examined with a fluorescence microscope. The slides were assigned a score of 5+ to 1+, according to the number of parasites observed: 5+ corresponds to >10 parasites per microscopic field (magnification, ×200), 4+ corresponds to 1 to 10 parasites per microscopic field, 3+ corresponds to >50 parasites per coverslip, 2+ corresponds to 10 to 50 parasites per coverslip, and 1+ corresponds to <10 parasites per coverslip. Samples were stored at 4°C until analysis.

Over the course of a 10-year period, 72 toxoplasma-positive samples were obtained by this method. Forty-eight of the 72 samples were obtained from AIDS

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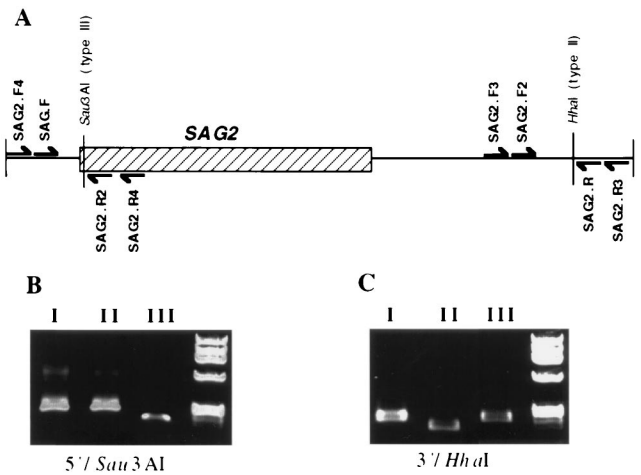

FIG. 1. Design of *SAG2* nested PCR analysis. (A) Schematic of the *SAG2* locus showing the locations of the primers used for nested PCR amplification of the 5′ and 3′ ends of the locus and the polymorphic restriction sites used for identification of strain genotypes. The hatched box represents the open reading frame of the *SAG2* gene. (B) *Sau*3AI restriction analysis of the 5′ amplification products from type I, II, and III strains. (C) *Hha*I restriction analysis of the 3′ amplification products from type I, II, and III strains. Products were resolved in 1.2% agarose gels stained with ethidium bromide. Molecular weight markers correspond to ϕX174 digested with *Hae*III.

patients, 10 samples were obtained from immunosuppressed patients (six bone marrow transplants, two heart transplants, one leukemia, and one allograft), and 14 samples were obtained from congenital infections. All of the congenital isolates were obtained from amniotic fluids. Samples from immunosuppressed patients were obtained from the following sources: one CSF, two heart biopsy, one brain biopsy, and six blood leukocyte samples. Samples from AIDS patients were obtained from the following sources: 10 bronchoalveolar lavage fluid, 2 bone marrow, 1 CSF, and 35 blood leukocyte samples.

Isolation of DNA. To isolate DNA from the fixed slides, the coverslips were flooded with water to dissolve the mounting medium and transferred to 24-well plates. DNA was purified by using a QIAamp blood kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's protocol. Briefly, the cells were lysed and digested with 1.8 mg of Qiagen's protease per ml for 10 min at 70°C, after which the samples were applied to DNA purification spin columns. The columns were washed twice, and the DNA was eluted from the columns with 50 μl of preheated (70°C) water. A 25-μl aliquot of the sample was used as the template in each of the two PCRs (described below).

Genotype analysis. Samples were analyzed at the *SAG2* locus by using a nested PCR approach that separately amplified the 5′ and 3′ ends of the locus (Fig. 1A) [14]. The 5′ end of the locus was amplified by standard PCR for 40 cycles with the primers SAG2.F4 (5′GCTACCTCGAACAGGAACAC3′) and SAG2.R4 (5′GCATCAACAGTCTTCGTTGC3′) at an annealing temperature of 65°C. The resulting amplification products were diluted 1/10 in water, and a second amplification of 40 cycles was performed with the internal primers SAG2.F (5′GAAATGTTTCAGGTTGCTGC3′) and SAG2.R2 (5′GCAAGAGCGAACTTGAACAC3′) by using 1 μl of the diluted product as the template. The amplified fragments were purified with GeneClean (Bio 101, Inc., Vista, Calif.) and digested with *Sau*3AI, and the restriction fragments were analyzed by agarose gel electrophoresis.

The 3′ end of the locus was similarly analyzed with the primers SAG2.F3 (5′TCTGTTCTCCGAAGTGACTCC3′) and SAG2.R3 (5′TCAAAGCGTGCATTATCGC3′) for the initial amplifications and the internal primers SAG2.F2 (5′ATTCTCATGCCTCCGCTTC3′) and SAG2.R (5′AACGTTTCACGAAGGCACAC3′) for the second round of amplification at an annealing temperature of 63°C. The resulting amplification products were purified with GeneClean, digested with *Hha*I, and analyzed by agarose gel electrophoresis.

RESULTS

We have previously described two independent RFLPs at opposite ends of the *SAG2* locus that, together, can unambiguously assign *T. gondii* strains to one of three separate lineages (6). We developed a nested PCR approach to detect and genotype low numbers of parasites in preserved tissues (Fig. 1A). To improve the sensitivity of PCR detection, the target regions for amplification were shortened to encompass only the relevant polymorphisms. Primers were selected to separately amplify the 5′ and 3′ ends of the *T. gondii SAG2* locus as 241-bp and 221-bp products, respectively. Digestion of the 5′ amplification products with *Sau*3AI distinguished allele 3 (type III strains) from alleles 1 and 2 (type I and II strains) (Fig. 1B), and digestion of the 3′ amplification products with *Hha*I distinguished allele 2 (type II strains) from alleles 1 and 3 (type I and III strains) (Fig. 1C).

To test the sensitivity of the nested PCR assay, normal host tissues were spiked with *T. gondii* parasites and used for nested PCR amplifications. *SAG2* was readily amplified from samples of HFF cells that were spiked with 25 or 5 parasites, while no product was detected from control cultures to which no parasites were added (Fig. 2A). Likewise, *SAG2* was successfully amplified when as few as five parasites were spiked into normal human CSF (Fig. 2B). In the absence of added parasites, this CSF sample was reproducibly negative by nested *SAG2* PCR (data not shown). Amplification of *SAG2* was slightly less sensitive in the presence of mouse brain tissue, and a positive signal was detected only from samples spiked with 25 parasites (Fig. 2B). Negative controls remained free of amplified products (Fig. 2 and data not shown).

A collection of fixed culture slides containing parasites from 72 unrelated toxoplasmosis patients were analyzed in this study. The number of parasites observed on most of the slides was low, with 52 (72%) of the slides scored only 1+ or 2+ (corresponding to fewer than 50 parasites in a background of approximately 10^5 host cells). Despite the low number of parasites on many of the slides, amplification of both the 5′ and 3′ ends of the *SAG2* locus from 68 of the 72 samples was successful. The four slides that were untypeable were scored 1+ or 2+ and were derived from three AIDS cases (two disseminated and one pneumonitis) and one congenital case. The failure to amplify the *SAG2* locus from these samples was presumably a result of too few parasites or poor sample preservation.

No mixed infections were detected, and all 68 of the successfully amplified samples were assigned to one of three distinct lineages based on *SAG2* alleles detected by RFLP analysis. Most of the strains, including 55 (81%) of the 68 samples, belonged to the type II lineage (Table 1). Type I strains were found in seven samples (10%), and type III strains were found

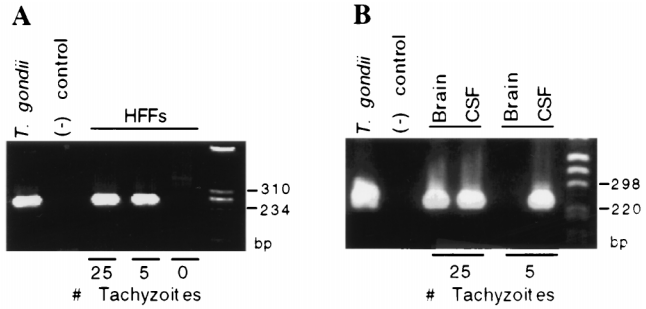

FIG. 2. Nested PCR amplification of *SAG2* from control tissues spiked with parasites. Positive amplifications were obtained from HFF (A) or human CSF (B) samples spiked with 5 or 25 freshly isolated parasites. The sensitivity of detection was slightly lower in the presence of normal mouse brain tissue (Brain lanes), where a positive signal was detected only for samples spiked with 25 parasites. The 5′ end of *SAG2* was amplified by nested PCR as described in Materials and Methods, and products were resolved on 1.2% agarose gels stained with ethidium bromide. Molecular weight markers correspond to ϕX174 digested with *Hae*III (A) and a lambda 1-kb ladder (B). The negative control in panel A corresponds to a water blank, while that in panel B corresponds to normal mouse brain in the absence of added parasites.

in six samples (9%). Of the 45 samples from AIDS patients successfully analyzed, 34 (76%) contained a type II strain of *T. gondii*. All 13 of the samples from cases of congenital toxoplasmosis contained a type II strain, while 6 of the 10 samples from non-AIDS immunosuppressed patients were found to contain type II strains. No correlations were apparent between the genotype of parasite strains and clinical presentation or the slide score based on in vitro growth (data not shown). There was also no correlation between the genotype of the parasites detected and the source of the clinical samples (i.e., bronchoalveolar lavage, biopsy, or blood leukocytes) (data not shown).

TABLE 1. Prevalence of *T. gondii* strain types in clinical samples

Clinical condition (no. of samples)	No. (%) of isolates		
	Type I	Type II	Type III
AIDS (45)			
Encephalitis	0	3	1
Pneumonitis	2	4	2
Disseminated infection	3	17	2
Unclassified infection	1	10	0
Total	6 (13)	34 (76)	5 (11)
Non-AIDS immunosuppression (10)	1 (10)	8 (80)	1 (10)
Congenital infection (13)	0 (0)	13 (100)	0 (0)
Total (68)	7 (10)	55 (81)	6 (9)

DISCUSSION

In the present report, we describe a rapid and efficient protocol for determining the genotype of *T. gondii* strains isolated from congenital, cerebral, and disseminated toxoplasmosis by short-term growth on MRC5 coverslip monolayers. Nested PCR amplification of the *SAG2* locus, followed by RFLP analysis, allowed assignment of all samples to one of three specific lineages of *T. gondii*. Type II strains of *T. gondii* were most often encountered in these samples, providing further evidence that strains of this genotype cause the majority of toxoplasmosis in humans.

PCR has previously been used for detection of *T. gondii* in clinical samples from patients with toxoplasmosis (18). Most investigators have used the *B1* or *SAG1* gene for detection (18); however, these loci are not sufficiently polymorphic to allow strain typing (6, 6a, 15). Consequently, we chose to develop a nested PCR assay based on the polymorphic *SAG2* locus (6). This gene is ideally suited for rapid genotyping, as it contains multiple lineage-specific polymorphisms. *SAG2* encodes two separate forms of the surface tachyzoite protein p22 that are recognized by strain-specific monoclonal antibodies: type I and III strains share the same protein allele, while type II strains have a second, distinct form (5, 12). Additional polymorphisms are present at the DNA level, allowing all three clonal genotypes to be rapidly identified by RFLP analysis at this single locus (6). Relying on a single marker for genotyping is generally not possible; however, in this case it is supported by the unusual population structure of *T. gondii* (1, 6, 15). In a previous study of 106 strains of *T. gondii*, multilocus genotyping revealed a highly clonal population consisting of only three major lineages (types I, II, and III) (6). Extensive linkage disequilibrium is a common characteristic of clonal populations, allowing a single locus to serve as a surrogate marker for the strain type. At the *SAG2* locus, only three alleles were found among the 106 isolates of *T. gondii*, and the allele carried at the *SAG2* locus was indicative of the strain type in 103 (97%) of the 106 strains (i.e., alleles 1, 2, and 3 represent strain types I, II, and III, respectively). Therefore, the *T. gondii* strain genotype can be determined with a high degree of confidence by identification of the *SAG2* allele.

In the present study, parasites were initially isolated from patient material (i.e., blood, CSF, and bronchoalveolar lavage fluid) prior to initiation of therapy by using a cell culture method combined with specific detection of *T. gondii* by immunofluorescence (2). In retrospect, we have performed nested PCR detection of the *SAG2* locus on these same samples, confirming the identification of *T. gondii* in 68 of 72 cases. Among the 68 cases of human toxoplasmosis analyzed here, type II strains were found in almost 80% of all cases and were therefore much more prevalent than type I or III strains (Table 1). This result supports previous findings that type II strains are most often associated with human toxoplasmosis (1, 6). Some type II strains produce high numbers of cysts during chronic infection in mice (11) and can cause toxoplasmic encephalitis in some strains of mice (8, 16). Both of these traits may lead to a greater chronic cyst burden that could favor reactivation in immunocompromised patients, such as organ transplant recipients and AIDS patients. However, it is not clear why type II strains are so often associated with human congenital toxoplasmosis, which is caused by primary infections of the mother which can pass across the placenta to the developing fetus.

The high prevalence of type II strains in human toxoplasmosis may simply reflect the source of strains that lead to human infection. We have previously reported that chronic infections of domestic and wild animals were equally split between type II and III strains (6). However, recent studies involving larger numbers of strains isolated from agricultural food animals indicate a high prevalence of type II strains in animals such as pigs in the United States (11a) and sheep in the United Kingdom (17a). These recent studies indicate a potentially important epidemiological link between chronic infections in some food animals that may underlie the prevalence of *T. gondii* genotypes causing human disease.

The nested *SAG2* PCR assay described here is highly sensitive, being able to detect as few as five parasites in samples that also contain mammalian genomes. Consequently, it should also be possible to use this analysis on primary clinical samples to detect toxoplasmosis. Nested *SAG2* PCR analysis has the advantage of also providing rapid, unambiguous assignment of a parasite genotype by RFLP analysis. The nested PCR assay makes possible additional retrospective studies on clinical samples that have been archived over the years in different laboratories; these studies are important, as they will provide a better understanding of the association between parasite genotypes and human toxoplasmosis.

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REFERENCES

1. **Dardé, M. L., B. Bouteille, and M. Pestre-Alexandre.** 1992. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates: biological and epidemiological implications. J. Parasitol. **78:**786–794.
2. **Derouin, F., C. Sarfati, B. Beauvais, M. C. Iliou, L. Dehen, and M. Lariviere.** 1989. Laboratory diagnosis of pulmonary toxoplasmosis in patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **27:**1661–1663.
3. **Dubey, J. P., and C. P. Beattie.** 1988. Toxoplasmosis of animals and man. CRC Press, Inc., Boca Raton, Fla.
4. **Dubey, J. P., and J. F. Frenkel.** 1972. Cyst-induced toxoplasmosis in cats. J. Protozool. **19:**155–177.
5. **Gross, U., W. A. Muller, S. Knapp, and J. Heesemann.** 1991. Identification of a virulence-associated antigen of *Toxoplasma gondii* by use of a mouse monoclonal antibody. Infect. Immun. **59:**4511–4516.
6. **Howe, D. K., and L. D. Sibley.** 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. **172:**1561–1566.

6a. **Howe, D. K., and L. D. Sibley.** Unpublished data.

7. **Howe, D. K., B. C. Summers, and L. D. Sibley.** 1996. Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. Infect. Immun. **64:**5193–5198.
8. **Hunter, C. A., and J. S. Remington.** 1994. Immunopathogenesis of toxoplasmic encephalitis. J. Infect. Dis. **170:**1057–1067.
9. **Israelski, D. M., and J. S. Remington.** 1993. Toxoplasmosis in the non-AIDS immunocompromised host. Curr. Clin. Top. Infect. Dis. **13:**322–356.
10. **Luft, B. J., and J. S. Remington.** 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. **15:**211–222.
11. **McLeod, R., E. Skamene, C. R. Brown, P. B. Eisenhauer, and D. G. Mack.** 1989. Genetic regulation of early survival and cyst number after peroral *Toxoplasma gondii* infection of AXB/BXA recombinant inbred and congenic mice. J. Immunol. **143:**3031–3034.

11a. **Mondragon, R., D. Howe, J. P. Dubey, and L. D. Sibley.** Unpublished data.

12. **Parmley, S. F., U. Gross, A. Sucharczuk, T. Windeck, G. D. Sgarlato, and J. S. Remington.** 1994. Two alleles of the gene encoding surface antigen P22 in 25 strains of *Toxoplasma gondii*. J. Parasitol. **80:**293–301.
13. **Pfefferkorn, E. R., and L. H. Kasper.** 1983. *Toxoplasma gondii*: genetic crosses reveal phenotypic suppression of hydroxyurea resistance by fluorodeoxyuridine resistance. Exp. Parasitol. **55:**207–218.
14. **Prince, J. B., K. L. Auer, J. Huskinson, S. F. Parmley, F. G. Araujo, and J. S. Remington.** 1990. Cloning, expression, and cDNA sequence of surface antigen p22 from *Toxoplasma gondii*. Mol. Biochem. Parasitol. **43:**97–106.
15. **Sibley, L. D., and J. C. Boothroyd.** 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature (London) **359:**82–85.
16. **Suzuki, Y., K. Joh, M. A. Orellana, F. K. Conley, and J. S. Remington.** 1991. A gene(s) within the H-2D region determines the development of toxoplasmic encephalitis in mice. Immunology **74:**732–739.
17. **Suzuki, Y., S. Y. Wong, F. C. Grumet, J. Fessel, J. G. Montoya, A. R. Zolopa, A. Portmore, F. Schumacher-Perdreau, M. Schrappe, and S. Koppen.** 1996. Evidence for genetic regulation of susceptibility to toxoplasmic encephalitis in AIDS patients. J. Infect. Dis. **173:**265–268.

17a. **Trees, A., and L. D. Sibley.** Unpublished data.

18. **Weiss, J. B.** 1995. DNA probes and PCR for diagnosis of parasitic infections. Clin. Microbiol. Rev. **8:**113–130.
19. **Wong, S., and J. S. Remington.** 1994. Toxoplasmosis in pregnancy. Clin. Infect. Dis. **18:**853–862.