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Microsatellite in the Beta-Tubulin Gene of *Toxoplasma gondii* as a New Genetic Marker for Use in Direct Screening of Amniotic Fluids

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To examine the correlation between *Toxoplasma gondii* genotype and congenital human toxoplasmosis, the polymorphism of the microsatellite consisting of a dinucleotide (TG) repeat in the intron of the beta-tubulin gene was investigated by PCR. Thirty-four reference strains were studied, including 7 strains virulent in mice and 27 strains avirulent in mice. The seven virulent strains had a (TG)8 microsatellite, and the avirulent strains had a (TG)7 microsatellite. This confirms the dichotomy already observed for virulent and avirulent strains. Additionally, 37 samples of amniotic fluid from infected fetuses were tested. All of them had the (TG)7 microsatellite marker. This result confirms that most of the human cases of congenital toxoplasmosis are due to strains avirulent in mice. Nevertheless, their virulence in human fetuses was obvious, as numerous abnormalities were observed on ultrasonic examination. The new genetic marker is the first one directly used for typing *T. gondii* isolates without any bias due to cultivation of the parasite. This microsatellite marker is not sufficient to type the strains which are avirulent in mice; however, seeking more polymorphic microsatellites should be worthwhile to obtain new genetic markers for direct screening of biological samples.

While normally benign, toxoplasmosis is life threatening in immunocompromised patients, especially in AIDS patients. The other main risk, recognized for more than half a century, is infection of the fetus if the mother contracts acute toxoplasmosis during pregnancy. However, not all acute infections lead to congenital disease; the risk and severity of infection in the fetus depends upon the point during pregnancy at which the mother acquires the infection. The earlier the infection during the pregnancy, the lower the fetal transmission rate; however, congenital toxoplasmosis is more severe in early infections (4, 8). Additionally, there is the possibility that both host and parasite factors could contribute to the pathogenicity. Indeed, there are some loci that control susceptibility to infection in mice (7) and in AIDS patients (21). On the other hand, underlying genetic factors specific to *Toxoplasma gondii* strains could influence the course of toxoplasmosis.

Genetic variations among *T. gondii* isolates were first suggested by differences in their pathogenicities in Swiss mice. Due to the worldwide distribution of the parasite, the broad host range of this species, and its capacity for sexual reproduction, a large genetic polymorphism was expected. Isoenzymatic studies first confirmed the existence of this genetic polymorphism, with three main zymodemes described (5, 6). Recently, some authors have described three major clonal lineages, types I to III, determined by multilocus restriction fragment length polymorphism analysis (13). Taken together with immunologic studies (17), these results suggest that zymodeme 1 (Z1) could be equated to genetic type I, Z2 to Z4 could be equated to type II, and Z3 could be equated to type III. A strong association was found between Z1 and acute virulence in mice (13, 20). A widely held definition of virulence is lethality to mice within 10 days when mice are injected with fewer than 10^4 tachyzoites. The virulent strains were more frequently found in patients with congenital toxoplasmosis than in animals (13). The hypothesis is that Z1 is responsible for higher parasitemia, which would increase the risk of transplacental transmission or the severity of the infection in a developing fetus (13, 14).

Correlation of *T. gondii* phenotype with the clinical presentation of congenital toxoplasmosis by using zymodemes or virulence in mice is hampered by the need to obtain live parasites. To screen human samples easily, PCR is more appropriate, as there is no need for cultivation of the parasite. We decided, therefore, to look for microsatellites, defined as short tandem repeats of two to six nucleotides, in the *T. gondii* genome. Microsatellites are known to be highly polymorphic as well as numerous and equally spread in the human genome (9) and are present in lower eukaryotes (10). From the technical point of view, the polymorphism of microsatellites can be evaluated by PCR and analysis with an automatic sequencer, which assumes reliability of the results. Once a polymorphic microsatellite was characterized, we screened amniotic fluid to determine the genotype responsible for congenital toxoplasmosis.

MATERIALS AND METHODS

Known *T. gondii* sequences were first subjected to an endogenous microsatellite search by using a program which permits the detection of repeated sequences containing at least five contiguous identical motifs of one to five nucleotides in length. A microsatellite DNA consisting of TG repeats was found in the first intron of the beta-tubulin gene (16). Primers were designed to amplify this sequence, and the polymorphism between reference strains was evaluated. The upper primer, 5′-CCAAGTTCTTCCGTCATTTC-3′, was located at position 698 (sense) (GenBank accession no. M20025). The lower primer, 5′-CCTCATTGT AGAACACATTGAT-3′, was located at position 806 (antisense). The upper primer was 5′-end labeled with fluorescein to allow sizing of PCR products with an automatic sequencer (see below).

Reference *T. gondii* strains, as determined by isoenzymatic analysis, were obtained as tachyzoites cultivated in mouse peritoneal exudate, purified from host cells, and then stored as frozen pellets at −80°C (6). DNA was extracted

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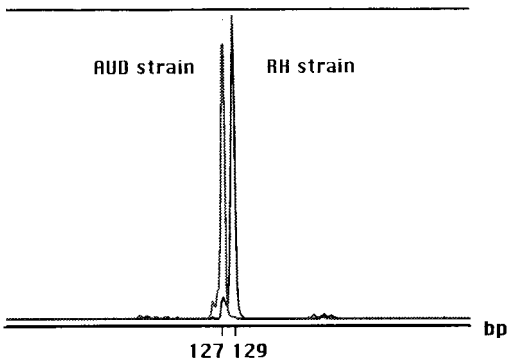

FIG. 1. Representative profiles obtained upon amplification with a fluorescein-labeled primer and analysis with an automatic sequencer by using the 672 GenScan software (Applied Biosystems). PCR products were run on an acrylamide urea gel with an internal standard labeled with 6-carboxy-X-rhodamine dye loaded in each well. The numbers refer to the sizes of the PCR products. Strains AUD (Z2 and avirulent in mice) and RH (Z1 and virulent in mice) were compared to show the difference in size between the (TG)7 repeat (strain AUD) and the (TG)8 repeat (RH strain).

from the frozen pellets with the QIAamp blood kit (Qiagen, Courtaboeuf, France). The amplification reaction mixture consisted of 100 ng of *T. gondii* DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 10 pmol of primers (DNAgency, Malvern, Pa.), and 1.25 U of *Thermophilus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Paris, France) in a 20-μl reaction volume. Hot start was achieved with a monoclonal antibody to *Taq* DNA polymerase (TaqStart antibody; Clontech, Palo Alto, Calif.). All amplifications were carried out with a Perkin-Elmer Cetus DNA thermal cycler and included 30 cycles of denaturation at 94°C and annealing at 55°C for 30 s each and extension at 72°C for 1 min. The final cycle was followed by an additional 7 min at 72°C to complete partial polymerization. PCR products were diluted 1/20 in water, and 1 μl of each of these was run on a 12-cm acrylamide-urea gel (6% acrylamide, 8.3 M urea, 1× Tris-borate-EDTA) for 2 h 30 min at 1,500 V. An internal standard labeled with 6-carboxy-X-rhodamine dye was loaded in each well along with the PCR products. Signals were read with an automatic sequencer (Applied Biosystems), and the data were stored and analyzed with 672 GenScan software (Applied Biosystems).

Thirty-seven samples of amniotic fluid known to contain *T. gondii* DNA from previous studies were tested (12). The fluid was processed as described elsewhere (12), and the amplifications were performed as described above. Ten fluid samples were obtained from pregnant females with *Toxoplasma* seroconversion during the first trimester; seven of the fetuses (70%) had severe abnormalities as determined by ultrasound examination at amniocentesis (two in utero deaths, three cerebral ventricular dilatations, and one case of hydrocephalus). Twenty fluid samples were obtained from mothers with seroconversion during the second trimester; four of the fetuses (20%) had ultrasonographic abnormalities (three cerebral ventricular dilatations and one intracerebral calcification). Seven fluid samples were obtained from mothers with seroconversion during the last trimester; one fetus (14%) had pleural effusion as determined by ultrasonography. Nineteen fluid samples (51%) came from Paris, France; 16 (43%) came from other parts of France or Switzerland; and 2 (5%) came from La Réunion in the Indian Ocean.

RESULTS

Of the 34 reference strains, 7 had been classified as Z1 and are virulent in mice (6, 13). The amplification of the beta-

TABLE 1. Characterization of the beta-tubulin genes of the 34 *T. gondii* reference strains tested

Strain	Origin	Initial host (infection or treatment)	Mouse virulence	Zymodeme	No. of TG repeats
BK	The Netherlands	Human (congenital[a])	Acute	1	8
CT-1	United States	Cow	Acute	1	8
ENT	France	Human (congenital)	Acute	1	8
FOU	France	Human (renal transplant)	Acute	1	8
GIL	France	Human (congenital)	Acute	1	8
P	France	Human (congenital)	Acute	1	8
RH	United States	Human (child)	Acute	1	8
AUD	France	Human (congenital)	Chronic	2	7
BEV	United Kingdom	Rabbit	Chronic	2	7
BOU.C	France	Human (AIDS)	Chronic	2	7
CAL	France	Human	Chronic	2	7
NTE	Germany	Human (AIDS)	Chronic	2	7
CHAM	France	Human (congenital)	Chronic	2	7
FAR.C	France	Human (congenital)	Chronic	2	7
FOUA	France	Human (congenital)	Chronic	2	7
JONE	United Kingdom	Human	Chronic	2	7
Me49	United States	Sheep	Chronic	2	7
PON	France	Human (congenital)	Chronic	2	7
PRE	France	Human (congenital)	Chronic	2	7
PRU	France	Human (congenital)	Chronic	2	7
REN	France	Human (congenital)	Chronic	2	7
ROD	France	Human (congenital)	Chronic	2	7
S1	France	Ovine aborted fetus	Chronic	2	7
S3.C	France	Ovine aborted fetus	Chronic	2	7
SUR	France	Human (AIDS)	Chronic	2	7
CEP	United States	Cat	Chronic	3	7
C56	United States	Chicken	Chronic	3	7
COR	France	Human (lymphoma)	Chronic	3	7
M7741	United States	Sheep	Chronic	3	7
OPA-OPA	Uruguay	Pig	Chronic	3	7
CHAMON	France	Human (congenital)	Chronic	4	7
DEG	France	Human (congenital)	Chronic	4	7
ELG	France	Human (AIDS)	Chronic	4	7
SQM	United Kingdom	Monkey	Chronic	4	7

[a] Congenitally infected with *T. gondii.*

tubulin genes of these seven strains gave a PCR product of 129 bp (Fig. 1). In contrast, the other 27 reference strains, which are avirulent in mice, yielded a 127-bp PCR product (Fig. 1). Sequencing of three PCR products of each length showed that the differences were due to one extra TG from strains of Z1. As the beta-tubulin gene is known to be single copy in the haploid *T. gondii* genome, each band was assigned to one allele. Thus, two alleles were defined: a (TG)8 repeat for the Z1 strains and a (TG)7 repeat for the other strains (Table 1). Results obtained upon amplification of two different DNA extractions of the same strain were identical to these results. In addition, the ts4 strain, an avirulent mutant of the virulent RH strain, had the same pattern as its parent strain, arguing for the stability of the microsatellite. Of the 37 samples of amniotic fluid tested, all had a (TG)7 microsatellite in the beta-tubulin gene.

DISCUSSION

The results obtained for the reference strains show that the TG microsatellite marker distinguishes the Z1 strains, virulent in mice, from others which are avirulent in mice. This is in perfect congruence with the dichotomy already observed with immunologic (2), enzymatic (1, 6, 14), or other (11, 13, 15, 17, 18) DNA markers. Like most of the others, the TG microsatellite marker is not particularly polymorphic, as only two alleles were found. However, the TG microsatellite could be added to other genetic markers since the polymorphism observed with microsatellites is based on a mechanism completely different from the one observed with the markers previously described (11, 13, 15, 17, 18) and is therefore complementary. With the latter, polymorphism depends on mutations in DNA sequences which can lead to the appearance or disappearance of restriction enzyme sites (13, 17) or which can be assessed by sequencing (15, 18). In contrast, for microsatellite polymorphism, the predominant method by which alleles of new lengths are generated is thought to be intra-allelic polymerase slippage during replication (19). It is the accumulation of length mutations which renders microsatellites among the most variable classes of repetitive DNAs. However, the more numerous the repeats, the more polymorphic the microsatellites (3, 22). It is, therefore, not surprising that only two alleles of seven and eight repeats were found in the beta-tubulin gene of *T. gondii*, as the threshold generally required for a human microsatellite to be considered polymorphic is 12 (22). It can be expected that in studying longer microsatellites, more polymorphic markers might become available for typing *T. gondii* strains.

The present study of amniotic fluid is the first one performed directly on biological samples without any bias due to parasite cultivation. The results for the 37 samples of amniotic fluid show that no congenital toxoplasmosis was caused by a strain with a mouse-virulent genotype. This observation reinforces previous studies based on clinical isolates or on laboratory strains showing that most of the isolates responsible for human symptomatic toxoplasmosis, whether in AIDS patients or in fetuses, belong to the avirulent-strain group (6, 13). However, the mouse-virulent strains have been described as being more frequent in humans with congenital toxoplasmosis than in animals (13). This observation might be due to a sampling bias because of the trend among medical parasitologists to select the mouse-virulent strains of the Z1 group. Indeed, Z1 strains are simply more likely to be sent to central laboratories which perform phenotypic and genotypic analysis because of their rarity. Besides, because most of the amniotic fluids were obtained from patients living in France, amniotic fluids from patients living in other countries should be tested to avoid a geographical bias.

The present results confirm that the main factor for the severity of congenital infection remains the stage of pregnancy at the time of contamination. Seventy percent of the fetuses infected during the first trimester had ultrasonic signs of advanced damage, in contrast with 20 and 14% during the second and third trimesters, respectively. This result is in complete accordance with previous studies showing the danger of early fetal contamination during pregnancy (4, 8, 12). However, it always remains possible that genetic diversity among the strains avirulent in mice may be responsible for part of the pathogenicity observed. To determine whether this is the case, other genetic markers are needed to screen clinical samples. The microsatellite in the beta-tubulin gene is not polymorphic enough for this purpose, but we think that other microsatellites, in particular, longer microsatellites, could be appropriate.

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