Rapid Identification of Virulent Type I Strains of the Protozoan Pathogen *Toxoplasma gondii* by PCR-Restriction Fragment Length Polymorphism Analysis at the B1 Gene

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Toxoplasmosis is a potentially fatal disease of the developing human fetus and immunocompromised (e.g., AIDS and transplant) patients and can cause severe ocular disease in otherwise healthy individuals (7, 10, 14). Recent population genetic studies have identified a remarkably limited number of *Toxoplasma gondii* genotypes in nature, the vast majority of which fall into one of only three distinct lineages (9). One of these lineages (type I) is highly virulent in mice (13). It is unclear whether a similar situation exists for human infection as inadequate data exist correlating the genotype of *Toxoplasma* with the symptoms and severity of disease that result. Studies to date have been hampered by the lack of a serological test to discriminate between strains and by insufficient parasite numbers in biopsy material for direct PCR amplification of single-copy polymorphic loci. Nevertheless, preliminary indications are that type II strains predominate in infections of immunocompromised patients and that type I strains are relatively overrepresented in congenital infections (8). More recently, we have uncovered a striking bias toward type I or type I-like strains associated with severe and/or atypical ocular toxoplasmosis in infected immunocompetent adults (M. E. Grigg, J. Ganatra, J. C. Boothroyd, and T. P. Margolis, submitted for publication).

*B1* is a tandemly arrayed 35-fold-repetitive gene routinely used for the highly specific and sensitive PCR detection of *Toxoplasma gondii* present in clinical specimens (3–6, 11, 12). Given its widespread use for diagnosis of *T. gondii* infection, we explored whether this locus could be used for genotyping the strains responsible for causing disease in infected individuals. We envisaged that if concerted evolution operates on the *B1* locus, all 35 *B1* genes should be highly conserved within a given strain, yet perhaps differ between strains, and thus make it possible to determine strain type in raw isolates where parasite DNA exists in vanishing amounts.

PCR primers were designed to amplify a large portion (−2 kb) of each *B1* gene repeat from archetypal type I, II, and III lineage strains to maximize our chances of identifying relevant polymorphisms that encode diagnostic restriction fragment length polymorphisms (RFLPs). For this study, we used the following well-characterized strains: RH (type I, mouse virulent), Prugniaud or PDS (type II, mouse avirulent), and CEP (type III, mouse avirulent). PCR amplification was carried out on 10³ parasite equivalents of either purified genomic DNA (prepared as described in reference 1) or cell lysate (prepared as described in reference 13). Each PCR utilized 5 µl of PCR Buffer (10× Perkin-Elmer PCR buffer containing 15 mM MgCl₂), 0.1 mM deoxynucleoside triphosphate mix, 10 pmol of each primer, and 1.5 U of *Taq* DNA polymerase in a total reaction volume of 50 µl. Each of the 30 cycles consisted of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. PCR amplification was performed using an automated thermocycler (Perkin-Elmer 140). PCR products were visualized using an ethidium-stained 0.8% agarose gel.

Sequence analysis was performed by the Stanford PAN facility on gel-purified DNA (UltraClean 15 DNA Purification kit; MoBio Labs) from the PCR-amplified material to ensure that the sequence obtained accurately reflects the nucleotide(s) present in most or all 35 *B1* genes. Comparison of the DNA sequence for *B1*-amplified material from each of the three strains identifies only 10 polymorphic sites. This confirms that there exists remarkable sequence conservation (~99.5%) regardless of the archetypal strain examined among all *B1* genes (Fig. 1A). All 10 polymorphic sites distinguish the virulent RH strain from the two avirulent strains, which gave the same sequence. At four of the polymorphic sites, a single, homogeneous nucleotide substitution is present in the RH repeats compared with the other two. For the remaining six sites, demarcated by an asterisk in Fig. 1A, a substantial and reproducible number of the *B1* gene repeats in a given strain possess one or the other of two nucleotides. Note that the relative ratio of nucleotides at these two-nucleotide sites varies somewhat between amplifications, as evidenced by multiple sequencing reads, but that there is always a substantial representation of both (Fig. 1B; data not shown).

Three of the six two-nucleotide sites fall within potential...
were subjected to lent lineages, seven type I, seven type II, and six type III strains determined by sequencing are in fact universal among avirulent strains (Fig. 2A). To ascertain whether the diagnostic restriction sites digestion is observed for the virulent RH strain PCR product amplification products from the two avirulent strains, whereas no consensus digestion confirms that all three enzymes restriction sites for \( \text{Xho I} \) or type II/III-like (cut by \( \text{Pml I} \) but not \( \text{Xho I} \)) (data not shown). In the very rare strains that are not type I, II, or III, the RFLP pattern was either type I-like (not cut by \( \text{Xho I} \) nor \( \text{Pml I} \)) or type II/III-like (cut by \( \text{Xho I} \) and \( \text{Pml I} \)), or, for one, there was a novel pattern (cut by \( \text{Pml I} \) but not \( \text{Xho I} \)) (data not shown).

In conclusion, the \( B1 \) assay described should permit the rapid and sensitive detection of parasite material in clinical samples for routine genotyping of strains from patients infected with \( T. gondii \). As with any single-locus genotyping strategy, however, the rare, atypical strains in the population structure of \( T. gondii \) (reported to occur at \( \sim 5\% \) frequency [9]) will not be correctly assigned by this methodology. The recent success of amplifying \( B1 \) from the peripheral blood of individuals suffering from acute disease should likewise obviate the need for recovering parasite material by more invasive biopsy and/or make it possible to determine the genotype in biopsy material when the amount of parasite DNA is too small to detect polymorphic single-copy loci (2). Moreover, prenatal diagnosis of congenital Toxoplasma infection by \( B1 \) PCR amplification from amniotic fluid is a routinely performed procedure (6). Our assay should provide the diagnostic means to readily determine whether \( T. gondii \) infection is caused by a mouse-virulent or -avirulent strain and whether strain type influences disease outcome in humans. Such information will allow the aggressiveness of the treatment to be matched to the predicted severity of the disease.
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REFERENCES