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Genotyping of the protozoan pathogen *Toxoplasma gondii* using high-resolution melting analysis of the repeated *B1* gene

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ABSTRACT

Genetic studies of the protozoan parasite *Toxoplasma gondii* have identified three main distinct types according to virulence in some hosts. Several methods have been developed to differentiate genotypes currently dominated by microsatellite markers targeting single-copy loci. We analyzed the possibility of using the 35-fold repetitive *B1* gene via high-resolution melting (HRM) curve analysis. Sequencing of the *B1* gene of 14 reference strains (four Type I, six Type II, and four Type III strains) identified 18 single nucleotide polymorphisms (SNP). Primers were designed to amplify eight of them for HRM analysis and for relative quantification of each nucleotide variation using SNaPshot mini-sequencing. Genotyping with five microsatellite markers was performed for comparison. Two to four HRM profiles were obtained depending on the SNP tested. The differences observed relied on the different ratios of nucleotides at the SNP locus as evidenced via SNaPshot mini-sequencing. The three main lineages could be distinguished by using several HRM profiles. Some HRM profiles proved more informative than the analysis based on five microsatellite markers, showing additional differences in Type I and Type II strains. Using HRM analysis, we obtained at least an equally good discrimination of the main lineages than that based on five microsatellite markers.

1. Introduction

Toxoplasma gondii is a protozoan parasite responsible for different medical entities, from asymptomatic infections to severe debilitating conditions such as meningoencephalitis or miscarriage. Population genetic studies have identified three main distinct lineages (Howe and Sibley, 1995; Saeij et al., 2005), one of which (Type I) is highly virulent in mice (Sibley and Boothroyd, 1992). In addition to these main lineages, there are also atypical strains difficult to classify (Su et al., 2010). For epidemiological studies and for research to seek out associations between a given parasite and a clinical presentation, several genotyping methods have been developed (Su et al., 2010).

A convenient genotyping system for *T. gondii* is based on microsatellite markers. The first attempt targeting the *beta-tubulin* gene was very efficient for distinguishing between Type I and Type II strains (Costa et al., 1997). Several microsatellite markers have been described since then (Ajzenberg et al., 2005; Ajzenberg et al., 2010). Microsatellites are defined as stretches of short tandem repeats of two to five nucleotides, and the polymorphism relies on the number of repeats. After amplification using two primers flanking the repeats, the length of the PCR products is used in order to differentiate the genotypes. Therefore, the amplified loci must be single-copy loci so as to yield a unique PCR product to be analyzed using electrophoresis.

We analyzed the possibility of using the *T. gondii B1* gene for genotyping. The 35-fold repetitive *B1* locus has been routinely used for PCR detection of *T. gondii* in clinical specimens since the early 1990s (Bretagne et al., 1993; Hohlfeld et al., 1994). Sequence analysis has already identified three restriction sites capable of discriminating Type I from Type II or III *T. gondii* strains using PCR-restriction fragment length polymorphism analysis (Grigg and Boothroyd, 2001). Given this context, we investigated whether high-resolution melting (HRM) of DNA could be an additional method to investigate *B1* polymorphism. In HRM analysis, comparison of melting curves allows for determining whether different amplicons have different or identical sequences (Wittwer et al., 2003; Reed et al., 2007). HRM of amplified double-stranded DNA is a potentially simple solution for genotyping, mutation scanning, and sequence matching (Costa et al., 2010).

2. Material and methods

2.1. Strains of Toxoplasma gondii

For each lineage, Type I (RH, ENT, VEL, GT-1), Type II (B7, P123, DEG, H44, Me49, PIH), and Type III (CTG, VEG, STRL, C5) strains were used in the present study. DNA from 10^{+6} tachyzoites harvested in

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40 µl of PBS was extracted by using the Total Nucleic Acid Isolation kit (Roche Diagnostics, Meylan, France) on a MagNA Pure Compact apparatus according to the manufacturer's instructions. DNA was eluted with 100 µl of elution buffer leading to a solution of about 10^4 tachyzoites-equivalent DNA per µl.

2.2. B1 gene direct sequencing

The complete *B1* gene sequence (AF179871) was determined by analysis of six overlapping PCR products for each of the 14 strains (Table 1). Amplification was carried out in a 50 µl reaction mixture containing 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM each dNTP, 0.2 µM each primer (Sigma, Paris, France), and 1.25 U of Fast Start Taq DNA polymerase (Roche Diagnostics, Meylan, France). After an initial denaturation at 95 °C for 10 min, samples were amplified for 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min. Specific amplification products were purified and then sequenced bi-directionally using the Dideoxy Terminator cycle sequencing kit v3 protocol (Applied Biosystems, Courtaboeuf, France). The reaction products were run on an ABI PRISM 3130 Genetic Analyzer and analyzed with the Sequence analysis/Seqscape v2.1 software (Applied Biosystems, Courtaboeuf, France).

2.3. Genotyping of isolates by microsatellite analysis

The 14 strains were genotyped by analyzing five microsatellite markers (TUB2, W35, TgM-A, B18, and B17) as initially described, with a slightly modified primer design (Table 1) aimed at shortening the PCR fragment lengths in order to increase the PCR reaction sensitivity (Costa et al., 1997; Ajzenberg et al., 2005). Briefly, amplification was carried out in a 20 µl reaction mixture containing 2.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM each dNTP, 0.2 µM each primer (Sigma, Paris, France), and 1.25U of Fast Start Taq DNA polymerase (Roche Diagnostics, Meylan, France). After an initial denaturation step at 95 °C for 10 min, samples were amplified for 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and an extension at 72 °C for 1 min. Next, 1 µl of 1:50 diluted PCR product was mixed with 9 µl of HiDi Formamide containing Genescan-500 TAMRA Size Standard (Applied Biosystems, Courtaboeuf, France). After denaturation at 95 °C for 2 min, the reaction was analyzed on an ABI3130 genetic analyzer using Genescan Software (Applied Biosystems, Courtaboeuf, France).

2.4. High-resolution melting curve and SNaPshot mini-sequencing for single nucleotide polymorphism analysis of B1 gene

All single nucleotide polymorphisms (SNPs) were studied during a single independent PCR reaction, except for the SNPs at positions 94 and 114, which were both studied in a single PCR fragment. PCR was set up in a final volume of 20 µl with the High Resolution Melting Master Kit (Roche Diagnostics, Meylan, France), 3 mM MgCl2, and each primer (Sigma, Paris, France) at a concentration of 0.5 µM. The reaction mixture was initially incubated for 10-min steps at 95 °C. Amplification was performed for 45 cycles of denaturation (95 °C for 10 s; ramp rate, 4.4 °C/s), annealing (60 °C for 10 s; ramp rate, 2.2 °C/s), and extension (72 °C for 15 s; ramp rate, 4.4 °C/s) in a LightCycler® 480 Instrument (Roche Diagnostics, Meylan, France). HRM was then performed at 95 °C for 1 min, at 40 °C for 1 min, and with increasing temperature from 65 °C to 95 °C at the rate of 1 °C/s with 25 acquisitions per °C. The melting profiles were assessed using the LightCycler® 480 Gene Scanning Software. Melting curves were fluorescence-normalized and temperature-shifted (threshold at 5), and sensitivity of grouping was adjusted to 0.50.

PCR products were purified with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo) in order to remove un-incorporated dNTPs and primers, respectively. Next, 3 µl of each PCR reaction was incubated with 2 µl of ExoSAP-IT (USB Europe, Staufen, Germany) for 60 min at 37 °C and then for 18 min at 75 °C for enzyme inactivation. SNaPshot analysis was carried out using the SNaPshot Multiplex kit (Applied Biosystems, Courtaboeuf, France) for each of the eight examined SNPs. The reaction was performed on a ABI9700 thermocycler in a final volume of 10 µl containing 5 µl of SNaPshot multiplex ready reaction mix, 5 pmoles of primer, and 3 µl of purified PCR products. Cycling conditions were 25 cycles of denaturation at 90 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 30 s. Labeled extension products were then treated with 1 µl (1U) of SAP (USB Europe, Staufen, Germany) for 1 h at 37 °C followed by a 15-min heat inactivation at 75 °C; 1 µl of this reaction was mixed with 9 µl of

Table 1
Primers used in this study for sequence analysis of *B1* gene and microsatellite genotyping.

	Sequences	Size of PCR product
Fragment 1	TGB1SEQ1: 5′-GCGTCATTGCTGTTCTGTCC-3′	447 bp
	TGB1SEQ2: 5′-AGAGGCGAGGTGGCACATT-3′	
Fragment 2	TGB1SEQ3: 5′-GCTGGCAAATACAGGTGAAATG-3′	458 bp
	TGB1SEQ4: 5′-CGCAGCGACTTCTATCTCTGTG-3′	
Fragment 3	TGB1SEQ5: 5′-GATAGTTGACCACGAACGCTTTA-3′	481 bp
	TGB1SEQ6: 5′-ATGCTTCTGCACAAAGTGAAGTC-3′	
Fragment 4	TGB1SEQ7: 5′-GAGAAAATCGATGGTGTCACG-3′	449 bp
	TGB1SEQ8: 5′-GTTGCCAGTCCTCCTTGAGA-3′	
Fragment 5	TGB1SEQ9: 5′-GCACCTTTCGGACCTCAACA-3′	452 bp
	TGB1SEQ10: 5′-GATTCTGCGTGGTGGGCTC-3′	
Fragment 6	TGB1SEQ11: 5′-AGTTGGTGATGGTTGCCTCG-3′	440 bp
	TGB1SEQ12: 5′-GGTGCCCTAGACAGACAGCG-3′	

For microsatellites markers

Target	Sequences	Modification
TUB2	TGTUB1: 5′-CCAAGTTCTTCCGTCATTTCA-3′	5′FITC
	TGTUB2: 5′-GAACACATTGATTCTCTCCAGC-3′	none
W35	TGW351: 5′-CTCGCAGAGGAGGTTCACTG-3′	5′HEX
	TGW352: 5′-TTAAACGAAGGACATGCCG-3′	none
TgM-A	TGMYO1: 5′-CATCTGCCTCTGGGTTTCTC-3′	5′HEX
	TGMYO2: 5′-AAATGCGGATGGAAACTACC-3′	none
B18	TGB181: 5′-GCTCTTGCTGTACTCCGCTT-3′	5′FITC
	TGB182: 5′-ATAGTTCAGTCTCGGGTGAGG-3′	none
B17	TGB171: 5′-AAAGATAATGGCGCAGAACC-3′	5′FITC
	TGB172: 5′-TAGGGATAAGTTTCTCACAACG-3′	none

For analysis of SNPs in the *B1* gene

SNP Position	PCR pimers and SNaPshot probe	Size of PCR product
94	TG94 + 114HRM1: 5′′ GATGCACCCGCAGAAGAA-3′	86 bp
	TG94 + 114HRM2: 5′- GGAAGAACTCCGTTGCGATA-3′	
	TG94SNP: 5′- CCCGCAGAAGAAGGGC-3′	
114	TG94 + 114HRM1: 5′- GATGCACCCGCAGAAGAA-3′	86 bp
	TG94 + 114HRM2: 5′- GGAAGAACTCCGTTGCGATA-3′	
	TG94SNP: 5′- CAGAACAGCAATGACGCCTT-3′	
366	TG366HRM1: 5′- ACGTACGACATCGCATTCAA-3′	94 bp
	TG366HRM2: 5′- ATTCTTCAGCCGTCTTGTGG-3′	
	TG366SNP: 5′- CCAGCAGATCTCGTTCGTGTA-3′	
754	TG754HRM1: 5′- TGGGAATGAAAGAGACGCTAA-3′	122 bp
	TG754HRM2: 5′- CGACCAATCTGCGAATACAC-3′	
	TG754SNP: 5′- CAGTGACTGCAACCTATGCAAA-3′	
1434	TG1434HRM1: 5′- TCGTCTCCATTCCGTACAGTC-3′	94 bp
	TG1434HRM2: 5′- GGCGTCATCCTGCTATTGAG-3′	
	TG1434SNP: 5′- AAAGAGAACATTCCAGCAACTTCT-3′	
1558	TG1558HRM1: 5′- AGCAAACACCGACGAACTCT-3′	87 bp
	TG1558HRM2: 5′- ACGTCTCTGTATCATCTCTGCG-3′	
	TG1558SNP: 5′- CGCGCCATCACGAACA-3′	
1586	TG1586HRM1: 5′- CGCAGAGATGATACAGAGACGT-3′	98 bp
	TG1586HRM2: 5′- GGTTGTTGAGGTCCGAAAGG-3′	
	TG1586SNP: 5′- AGCGACCAACCTTGTCCTG-3′	
1592	TG1592HRM1: 5′- TTCCGGTCGAGAGGCTAAAC-3′	82 bp
	TG1592HRM2: 5′- GAGGCAACCATCACCAACTG-3′	
	TG1592SNP: 5′- TTTTGTTAAGCTGATGGCTGC-3′	