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Variation of *B1* Gene and AF146527 Repeat Element Copy Numbers According to *Toxoplasma gondii* Strains Assessed Using Real-Time Quantitative PCR

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Using the multicopy *B1* gene and AF146527 element for the amplification of *Toxoplasma gondii* DNA raises the issue of reliable quantification for clinical diagnosis. We applied relative quantification to reference strains using the single-copy *P30* gene as a reference. According to the parasite type, the copy numbers for the *B1* gene and AF146527 element were found to be 5 to 12 and 4 to 8 times lower than the previous estimations of 35 and 230 copies, respectively.

The diagnosis of diseases resulting from the protozoan parasite *Toxoplasma gondii* utilizes PCR for both immunocompromised patients (3, 17) and congenital diseases (9, 11, 12, 21). Over the past decade, PCR methods have evolved from endpoint PCR toward real-time quantitative PCR (qPCR), which is characterized by using a closed-tube method with a low risk of contamination with PCR products (2). This method provides quantitative results, which help clinicians in the management of congenital diseases (9) and *Toxoplasma* encephalitis (10, 17).

To improve sensitivity, the targeted sequence has developed from single-copy genes, such as *P30* (5), to the repeated *B1* gene (3, 11) and the 529-bp repeat element (AF146527) (13, 19). However, targeting multicopy genes has several issues. One challenge is deciphering the number of repeats for each strain, while another is choosing primers and probes based on conserved sequences from among the different repeats of the three major *T. gondii* lineages (20), with the aim of avoiding false negativity and quantification errors. We applied the E-method (22) to determine the relative quantification of the *B1* gene and AF146527 (or AF487550) (19) repeat element on several *T. gondii* reference strains. The E-method compensated for differences in target and reference gene amplification efficiency, thus reducing calculation errors compared with the threshold cycle ($\Delta\Delta C_T$) method (16).

Overall, 12 reference strains pertaining to the three different *T. gondii* types were used (Table 1). Total DNA was extracted from 40 μl of a suspension of 10^8 tachyzoites per ml using the Roche Diagnostics total nucleic acid isolation kit (Roche Diagnostics, Meylan, France) on a MagNA Pure compact apparatus and eluted with 100 μl of elution buffer.

Partial sequences of the *P30* gene, *B1* gene, and AF146527 repeat element were obtained for all strains using the conventional cycle-sequencing Sanger method (7). Primers for relative quantification were then selected using Oligo 6.0 software among 100% conserved regions and designed to obtain similar sizes for the amplified products in order to achieve a better balance in the amplification yield (Table 2). BLAST analysis of the *T. gondii* genome confirmed that each primer set targeted a single, specific locus.

To obtain the amplification efficiency for each DNA target and parasite type, PCRs were performed in triplicate as previously described (19), with serial 10-fold dilutions of *T. gondii* DNA ranging from 8×10^4 to 80 parasites per PCR, with one strain representing each type, namely, the RH, B7, and C5 strains for types I, II, and III, respectively. Calibration curves were calculated by plotting the quantification cycle values against the logarithm of input DNA.

DNA of each strain (2.5 μl containing 10^3 parasite equivalents per reaction) was amplified in duplicate in order to determine the copy number of the *B1* gene and AF146527 repeat element for each *T. gondii* strain. To avoid interrun variability, reactions were performed in the same multiwell plate in a single run. Relative quantification was calculated automatically using LightCycler 480 software based on the E-method, with the single-copy *P30* gene as a reference. Results were expressed as the ratio of target to reference sequence.

PCR efficiencies ranged from 1.878 to 1.989, depending on the combination of PCR targets and strain types. Thus, the relative quantifications of the *B1* gene and AF146527 repeat element were calculated for each strain based on the PCR efficiency of the corresponding type. The difference was found to be significant for the *B1*/*P30* and AF146527/*P30* ratios (Table 1). When comparing the results according to type, using the Mann-Whitney-Wilcoxon test, type 2 *B1*/*P30* ratios were found to be different from the other *B1*/*P30* ratios ($P < 0.03$), while the AF146527/*P30* ratios all differed from each other ($P < 0.03$). Whether this finding can enhance known genotyping methods or not still needs to be confirmed using an extended sampling.

The copy numbers for the *B1* gene were observed to be 5, 12, and 7 times lower than the previous estimation of 35 (4) for the three different *T. gondii* types, respectively, and at least 8, 4, and 4 times lower than the 200 to 300 copies reported for the AF146527 element (13). These discrepancies may be explained by the possible overestimation of the copy numbers in previous studies.

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TABLE 1 Mean ratios for relative quantification of the *B1* gene and AF146527 repeat element compared to the single-copy *P30* gene in different strains of *Toxoplasma gondii*[a]

	Type I			Type II			Type III			
		Mean (SD) ratio			Mean (SD) ratio			Mean (SD) ratio		
Gene copy ratio	Strain name	Strain	Type	Strain name	Strain	Type	Strain name	Strain	Type	*P* value[b]
B1/*P30*	RH	7.1 (0.7)	7.3 (2.0)	B7	2.5 (0.0)	2.9 (0.3)	STRL	4.0 (0.2)	4.9 (0.7)	0.01
	VEL	6.4 (0.4)		H44	2.7 (0.3)		VEG	4.9 (0.3)		
	ENT	10.2 (0.8)		Me49	3.1 (0.2)		C5	5.8 (0.5)		
	GT-1	5.7 (0.2)		PIH	3.2 (0.1)		CTG	4.9 (0.2)		
AF146527/*P30*	RH	30.4 (5.4)	24.3 (4.2)	B7	52.3 (5.5)	53.7 (10.2)	STRL	49.1 (2.6)	46.9 (6.2)	0.02
	VEL	23.8 (0.5)		H44	57.8 (3.2)		VEG	53.7 (5.9)		
	ENT	21.8 (1.1)		Me49	40.3 (2.6)		C5	39.0 (2.4)		
	GT-1	21.1 (0.6)		PIH	64.3 (1.6)		CTG	46.0 (4.8)		
AF146527/*B1*	RH	4.3 (0.7)	3.5 (0.9)	B7	20.7 (1.9)	18.8 (3.9)	STRL	12.4 (1.0)	9.9 (2.4)	0.01
	VEL	3.7 (0.3)		H44	21.3 (3.2)		VEG	11.1 (1.9)		
	ENT	2.1 (0.2)		Me49	13.0 (0.3)		C5	6.8 (0.4)		
	GT-1	3.7 (0.1)		PIH	20.0 (0.6)		CTG	9.4 (1.4)		

[a] *T. gondii* was harvested on human foreskin fibroblast culture, as described at http://sibleylab.wustl.edu/pdf/Toxo_Harvesting.pdf. Mean values are based on three independent experiments, with ratios calculated automatically using relative quantification software with qPCR efficiency specific for each *T. gondii* type.
[b] Kruskal-Wallis rank sum test, with $P < 0.05$ considered significant.

Quantification was historically performed with blotting following conventional PCR, and the signals were compared between quantified *T. gondii* DNA and a plasmid containing one copy of the gene (4, 13). Additionally, only type I *T. gondii* was tested (4, 13). These methods were subjective, with the quantification perhaps being only indicative. The discrepancies in copy numbers may also be accounted for by an underestimation based on the present relative quantification in relation to single nucleotide polymorphisms in the primer sequences between the different repeats. Indeed, Sanger's sequencing cannot detect sequence variations below a 20% threshold (14). However, this explanation appears unlikely.

While *B1* and AF146527 copy numbers were below the levels previously published, we confirmed the higher copy number of AF146527 over *B1*, although the ratio varied according to the different types (Table 1). This finding is in agreement with the higher sensitivity observed in clinical specimens, where between 9.1% and 18.5% of AF146527-positive samples were reported to be *B1* negative, supporting the use of the AF repeat element as the best target for a routine diagnostic (6, 15, 18). However, a recent publication performed on African patients suggested that the AF146527 locus cannot be amplified in certain samples (23). We reported a C/G mismatch at position 275 in one primer sequence under GenBank accession no. AF487550 (19), which may explain some of the amplification failures. Additionally, the strain types in Africa may differ from the common type II found in Europe (1, 8). Indeed, type II strains exhibit a higher AF146527/*B1* ratio, which may explain the improved performance of PCR assays based on AF146527 in Europe compared with other geographical regions.

Our study highlighted the relevance of relative quantification for correctly identifying reference strains when comparing the analytical sensitivity of PCR assays based on multicopy genes. As

TABLE 2 Primers and probes used in the present study

Primer or probe (GenBank accession no.)	Sequence	PCR product size (bp)	Modification
P30 gene (AY187278)		126	
P30-1	5′-AGTTCCAATCGAGAAGTTCCC-3′		None
P30-2	5′-TTATTGACGACCGATGAGGC-3′		None
P30-3	5′-CAACCGACCACAAACGTCTGCG-3′		5′LCRed640 3′Ph
P30-4	5′-CAACTCTGTGCGTCGTCTCCCTTGAT-3′		3′ FITC
B1 gene (AF179871)		132	
B1-1	5′-TCTCTCAAGGAGGACTGGCA-3′		None
B1-2	5′-GTTTCACCCGGACCGTTTAG-3′		None
B1-3	5′-ACGGGCGAGTAGCACCTGAGGAG-3′		5′LCRed640 3′Ph
B1-4	5′-CGGAAATAGAAAGCCATGAGGCACTCC-3′		3′ FITC[a]
AF146527 element (AF487550)		133	
CG-1	5′-GTTGGGAAGCGACGAGAGTC-3′		None
CG-2	5′-ATTCTCTCCGCCATCACCAC-3′		None
CG-3	5′-TGCGATCTAGACGAGACGACGCTTTC-3′		5′LCRed640 3′Ph
CG-4	5′-GGAGGGTGGAAAAGAGACACCGGA-3′		3′ FITC

[a] FITC, fluorescein isothiocyanate.

human infections are mainly due to non-type I strains, using the type I RH strain may be misleading in terms of quantitative results.

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