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Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: A high resolution and simple method for identification of parasites

C. Su [a,*], X. Zhang [a], J.P. Dubey [b]

[a] *Department of Microbiology, University of Tennessee, 1414 W. Cumberland Avenue, Knoxville, TN 37996-0845, USA*
[b] *Animal Parasitic Diseases Laboratory, ANRI, ARS, USDA, Beltsville, MD 20705, USA*

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Abstract

It was generally believed that *Toxoplasma gondii* had a clonal population structure with three predominant lineages, namely types I, II and III. This was largely based on genotyping of more than 100 *T. gondii* isolates originating from a variety of human and animal sources in North America and Europe. Recent genotyping studies on *T. gondii* strains from wild animals or human patients from different geographical regions revealed the high frequency of non-archetypal genotypes, suggesting the overall diversity of the *T. gondii* population might be much higher than we thought. However, as most genotyping studies had relied on a few biallelic markers, the resolution and discriminative power of identifying parasite isolates were quite low. To date, there is no commonly used set of markers to genotype *T. gondii* strains and it is not feasible to compare results from different laboratories. Here, we developed nine PCR-restriction fragment length polymorphism markers with each of them capable of distinguishing the three archetypal *T. gondii* alleles in one restriction-enzyme reaction by agarose gel electrophoresis. Genotyping 46 *T. gondii* isolates from different sources using these markers showed that they could readily distinguish the archetypal from atypical types and reveal the genetic diversity of the parasites. In addition, mixed strains in samples could be easily detected by these markers. Use of these markers will facilitate the identification of *T. gondii* isolates in epidemiological and population genetic studies.

Keywords: *Toxoplasma gondii*; PCR-RFLP; Multilocus genotyping; Genetic diversity

1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that is capable of infecting all warm-blooded vertebrates including mammals and birds. Up to one-third of the human population is chronically infected (Dubey and Beattie, 1988; Tenter et al., 2000). Human infections are primarily caused by ingesting uncooked meat containing viable *T. gondii* or by ingesting food or water contaminated with oocysts shed in the faeces of infected cats (Dubey, 2004). Most primary infections in adults are asymptomatic but in some patients lymphadenophathy or ocular toxoplasmosis can occur. Infection acquired during pregnancy may cause severe damage to the fetus. In immunocompromised patients, reactivation of latent infection can cause life-threatening encephalitis (Montoya and Liesenfeld, 2004).

Most *T. gondii* isolates from human and animal sources in Northern America and Europe have been grouped into one of three clonal lineages by multilocus enzymes electrophoresis, PCR-restriction fragment length polymorphism (RFLP) and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). Even though the differences among these three genotypes at the genome sequence level is less than 1%, they have strikingly different virulence phenotypes in mice, with type I strains uniformly lethal in outbred mice with $LD_{100}=1$; in contrast, type II and III strains are significantly less virulent with $LD_{100}\geq 10^3$ (Sibley and Boothroyd, 1992). It is of great interest to investigate if the genotypes are related to the disease manifestations in human toxoplasmosis. Some studies reported that type I strains were more frequently associated in congenital and ocular toxoplasmosis in human patients based on one marker at *SAG2* locus (Fuentes et al., 2001; Vallochi et al., 2005). However, we must be cautious about the interpretation of these studies, as the genetic marker used has limited power to distinguish parasite isolates. For those *T. gondii* isolates that do belong to the same

* Corresponding author. Tel.: +1 865 974 4015; fax: +1 865 974 4007.
E-mail address: csu1@utk.edu (C. Su).

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clonal lineage, biological information obtained from one representative may successfully predict the others because of their identical genetic background (high linkage disequilibrium). The same may not be true for strains with different genetic makeup, particularly when frequent genetic crosses occur in the population. In such cases, there is low linkage disequilibrium and the association of genotypes with phenotypes is diminished. Recent studies of nine *T. gondii* strains from human patients in French Guiana and 53 strains from chickens in Brazil suggested the high rate of transmission and out-crossing (Ajzenberg et al., 2004; Lehmann et al., 2004), in sharp contrast with what has been observed in *T. gondii* isolates from human patients and domestic animals in North America and Europe (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). In addition, there is increasing interest in the biological differences among different genotypes of *T. gondii* (Saeij et al., 2005). This highlights the need to develop a set of markers, which can provide higher resolution for *T. gondii* genotyping. Here, we developed nine PCR-RFLP markers with each of them capable of distinguishing the three alleles in one restriction-enzyme reaction by agarose gel electrophoresis. We used these markers to genotype 46 natural *T. gondii* isolates from different sources and the results showed that these markers could readily distinguish the clonal from non-clonal types and to reveal genetic diversity of the parasites with the resolution similar to DNA sequencing.

2. Materials and methods

2.1. Multilocus PCR-RFLP markers

The goal of this study was to develop a set of unlinked three-way markers so that each has the resolution to distinguish the three clonal lineages (type I, II and III) after PCR amplified targets are treated with one restriction enzyme or two enzymes by double digestion. At the DNA sequence level, *T. gondii* strains are essentially dimorphic in that for a given single nucleotide polymorphism (SNP) there are only two alleles. Most available genetic markers are biallelic and can only distinguish two of the three clonal types at a single locus. A small percentage of available markers have two biallelic polymorphisms that lie closely by each other and they can distinguish all three clonal types by using one or two restriction enzymes. From a collection of over 200 genetic markers in the *Toxoplasma* Genome Map Database (http://toxomap.wustl.edu/Toxo_Genetic_Map_Table.html), 12 unlinked three-way markers were tested by double digestion and analysed by agarose gel electrophoresis to ascertain whether the resolution was sufficient for typing. In addition, a three-way marker encoded by the apicoplast genome was also tested. For each candidate marker, the target DNA sequence was amplified by PCR using FastStart DNA polymerase (Roche). The reaction was carried out in 25 μl of volume containing 1×PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0.3 μM each of the forward and reverse primers, 0.75 units of FastStart DNA polymerase and 1.5 μl of DNA lysate ($\sim 10^7$ *T. gondii*/ml in lysate) of reference type I (RH88), II (PTG) or III (CTG) *T. gondii* strains. The reaction mixture was first treated at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 min, 55 °C for 1 min and 72 °C for 2 min. Five microliters of PCR products were examined by electrophoresis in 1.5% agarose gel containing 0.3 μg/ml ethidium bromide and visualised under UV light. To reveal the RFLP pattern of each reference strain, 3 μl of PCR products were mixed with 17 μl of digestion reaction containing 1×NEB buffer, 0.1 mg/ml BSA, 1 unit each of the two restriction enzymes. The reaction was carried out by incubating at the proper temperature for each restriction enzyme by the manufacturer's instruction (New England BioLab, Beverly, MA). The digested PCR products were resolved in a 2.5–3% agarose gel by electrophoresis in the presence of 0.3 μg/ml ethidium bromide and visualised under UV light. The markers that can differentiate the three reference strains without ambiguity were kept for genotyping in this study.

2.2. Toxoplasma gondii strains

To test the discriminatory power of the selected three-way markers, a total of 46 *T. gondii* isolates were genotyped. Of these isolates, 18 had been intensively studied through DNA sequencing (Grigg et al., 2001a; Su et al., 2003; Ajzenberg et al., 2004), another 28 were isolated from 58 domestic cats within a 3-km area in Paraná State, Brazil and previously genotyped at the *SAG2* locus (Dubey et al., 2004); these isolates have not undergone frequent passages in mice or cell culture. For the present study, the tachyzoites were derived from the oocysts excreted by experimental cats that were fed naturally-infected cat tissues (Dubey et al., 2004). The previously sequenced 18 isolates included four isolates of type I lineage (RH88, GT1, ENT and VEL), three isolates of type II lineage (PTG, DEG and PIH), three isolates of type III lineage (CTG, STRL and VEG), seven atypical isolates (CAST, COUGAR, MAS, CASTELLS, VAND, GUY-DOS, GUY-MAT) and GPHT (an I/III recombinant isolate). Most of these isolates had been maintained in various laboratories for unknown periods. Previous studies of multilocus DNA sequences in these 18 isolates identified little nucleotide polymorphism within the clonal lineages but abundant single nucleotide polymorphisms among each unique 'atypical' isolate (Grigg et al., 2001a; Su et al., 2003; Ajzenberg et al., 2004). These previously studied isolates served as the basis to test the newly developed three-way markers.

2.3. Toxoplasma gondii *DNA lysate preparation and genotyping*

Of the 46 *T. gondii* isolates used in this study, DNA lysates of four samples including GPHT, GUY-DOS, GUY-MAT and VAND were kindly provided by Dr David Sibley at the Washington University School of Medicine, St Louis, MO. DNA lysates for other isolates were prepared by growing the parasites in human foreskin fibroblast (HFF) cell culture (Roos et al., 1994). For the 28 Brazil cat isolates, oocysts (15 μl) collected a year earlier from experimental cats that were fed naturally-infected cat tissues (Dubey et al., 2004) were used to

Table 1
Summary for three-way markers

Marker	Chr.[a]	PCR primers	Size (bp)	Restriction enzymes	Enzyme digestion and electrophoresis	Reference
c22-8	Ib	c22-8F:TCTCTCTACGTG-GACGCC c22-8R:AGGTGCTTG-GATATTCGC	521	*BsmA* I, *Mbo* II	NEB2, BSA, 37 °C 30 min 55 °C 30 min 2.5% gel	Khan et al. (2005) and this study
c29-2	III	c29-2F:AGTTCTGCA-GAGTGTCGC c29-2R:TGTCTAGGAAAGAGGCGC	446	*HpyCH4*IV, *Rsa* I	NEB1, BSA, 37 °C 60 min 2.5% gel	Khan et al. (2005) and this study
L358	V	L358-F2:AGGAGGCG-TAGCGCAAGT L358-R2:CCCTCTGGCTGCAGTGCT	418	*Hae* III, *Nla* III	NEB4, BSA, 37 °C 60 min 2.5% gel	Khan et al. (2005) and this study
PK1[b]	VI	PK1-F:CGCAAAGGGAGA-CAATCAGT PK1-R:TCATCGCT-GAATCTCATTGC	903	*Ava* I, *Rsa* I	NEB4, BSA, 37 °C 60 min 2.5% gel	Khan et al. (2005) and this study
SAG2	VIII	SAG2-Fa:ACCCATCTGCGAA-GAAAACG SAG2-Ra:ATTTC-GACCAGCGGGAGCAC	546	*Hinf* I, *Taq* I	NEB3, BSA, 37 °C 30 min, 65 °C 30 min 2.5% gel	Lehmann et al. (2000) and this study
BTUB	IX	Btb-F:GAGGTCATCTCGGAC-GAACA Btb-R:TTGTAGGAA-CACCCGGACGC	411	*Bsi*E I, *Taq* I	NEB4, BSA, 60 °C for 60 min 2.5% gel	Khan et al. (2005) and this study
GRA6	X	GRA6-F1:TTTCCGAG-CAGGTGACCT GRA6-R1x:TCGCCGAAGAGTTGA-CATAG	344	*Mse* I	NEB2, BSA, 37 °C 60 min 2.5% gel	Fazaeli et al. (2000) and this study
SAG3	XII	P43S1:CAACTCTCAC-CATTCCACCC-3 P43AS1:GCGCGTTGTTAGA-CAAGACA	311	*Nci* I	NEB4, BSA, 37 °C 60 min 2.5% gel	Grigg et al. (2001b)
Apico	Plastid	Apico-F:TGCAAATTCTT-GAATTCTCAGTT Apico-R:GGGATTCGAACCCTTGATA	640	*Afl* II, *Dde* I	NEB2, BSA, 37 °C 60 min 3% gel	This study

[a] Chromosome number.
[b] Marker *PK1* is developed based on *Toxoplasma gondii* protein serine/threonine kinase (*PK1*) gene sequence (Ng et al., 1997).

infect a mouse by i.p. injection. Seven days after injection, tachyzoites were collected from mouse peritoneal lavage and inoculated to HFF cells. After the parasites had lysed HFF cells in a T25 flask (Corning Inc., Acton, MA), the tachyzoites were harvested by filtering through a 3 μm PCTE filter membrane (Fisher, Hanouver Park, IL) and spun down by centrifugation at 1800 rpm (800×*g*) for 10 min. The pellets were resuspended in 1 ml of PBS and 20 μl of 10 mg/ml of proteinase K were added. The mixtures were incubated at 55 °C for 2 h to lyse the parasites and release the DNA molecules. Proteinase K was then inactivated at 95 °C for 15 min. In general, the DNA lysates contained approximately 10^7 copies of genomic DNA per millilitre. For genotyping, the same protocol as testing the markers was used.

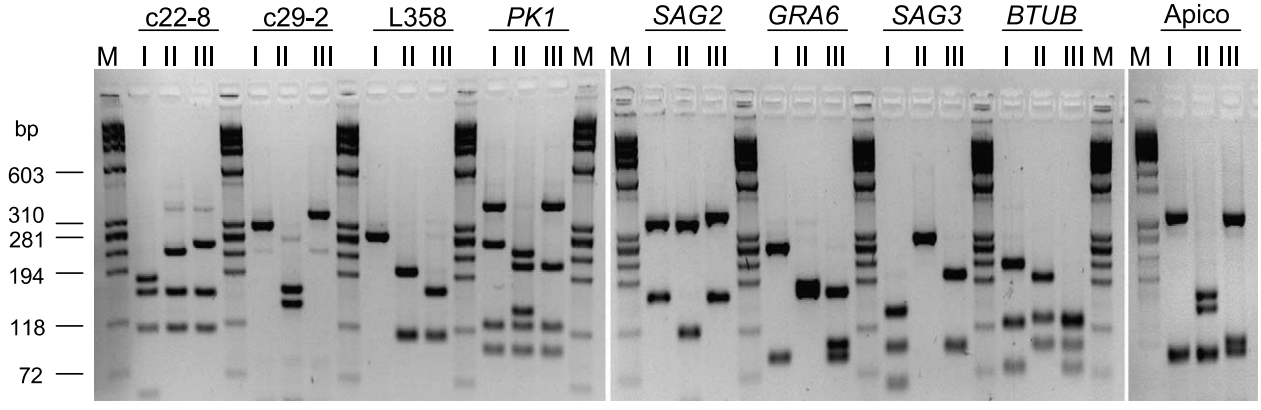

Fig. 1. Gel images for the nine three-way PCR-restriction fragment length polymorphism markers. Reference type I, II and III strains are RH88, PTG and CTG, respectively. DNA sequence for each marker was amplified by PCR. The PCR products were digested with corresponding restriction enzyme(s) and DNA fragments were separated in agarose gel. All markers except Apico were resolved in 2.5% agarose gel and Apico was resolved in 3% gel. M: DNA molecular marker phiX174 DNA-*Hae* III digest.

3. Results

3.1. Multilocus PCR-RFLP markers

A total of nine three-way markers residing on eight nuclear chromosomes and the apicoplast were developed (Table 1). Marker *SAG3* required only *Nci* I to discriminate the three archetypal alleles (Grigg et al., 2001b). *GRA6* requires only *Mse* I for typing and the PCR primers were designed based on the original report of its DNA sequence information (Fazaeli et al., 2000). The *SAG2* marker in this study was developed based on the sequences of *SAG2* gene from a number of *T. gondii* strains (Lehmann et al., 2000) but it was different from the previously published *SAG2* marker by Howe et al. (1997). The detailed information for chromosome localisation, PCR primers, PCR product size (base pair), restriction enzymes, conditions for enzyme digestion and agarose gel electrophoresis are summarised in Table 1. Product of each marker was presented in Fig. 1. The reference strains of clonal type I, II and III used for marker development were RH88, PTG and CTG, respectively. All eight markers on chromosomes were resolved in 2.5% agarose gel. Marker Apico for apicoplast genome was resolved in 3% gel. It was clear that all markers can distinguish the three clonal alleles without ambiguity.

3.2. Genotyping and data analysis

Genotyping results of the 46 *T. gondii* isolates at all nine loci are summarised in Table 2. The 10 clonal isolates had uniform typing patterns, except one for clonal type II, where a type I allele at Apico locus was identified for isolate DEG (Table 2). For each of the seven atypical and GPHT (previously identified with a combination of type I and III alleles) isolates, unique multilocus genotype with different combination of all three clonal alleles (I/II/III) at different loci resulted. In addition, a unique allele (u-1) was identified for isolates MAS and GPHT at locus c22-8. These results were in perfect agreement with previous multilocus DNA sequencing studies with regard to the power of identifying and differentiating these isolates (Grigg et al., 2001a; Su et al., 2003; Ajzenberg et al., 2004).

There were a few new alleles (non-archetypal I, II and III) identified by three of the nine three-way markers including PK1, c22-8 and c29-2. An example of genotyping at PK1 locus is presented in Fig. 2. Two unique alleles revealed by *PK1* were denoted as 'u-1' in isolates TgCatBr5, 9, 11, 16, 18, 19, 26 and as 'u-2' in isolate COUGAR (Fig. 2 and Table 2). The unique alleles revealed at locus c22-8 in isolates MAS, GPHT, TgCatBr2, 12, 17, 18, 21, 25, 30, 34 and locus c29-2 in isolate COUGAR were denoted as 'u-1'. All unique alleles were confirmed by replicate reactions of PCR-RFLP experiments.

Of the 28 Brazilian cat isolates, there were six genotypically unique isolates and another six genotypes characterising multiple isolates (Table 2). The six genotypes with multiple isolates were [TgCatBr1 and 7], [TgCatBr2, 12, 17, 21 and 30], [TgCatBr3 and 4], [TgCatBr5, 11 and 16], [TgCatBr9 and 19] and [TgCatBr10, 22, 23, 28, 31, 32 and 37], respectively. The six unique isolates identified were TgCatBr15, TgCatBr18, TgCatBr20, TgCatBr25, TgCatBr26 and TgCatBr34, respectively. The genotype of TgCatBr2 was identical to GPHT at all loci. Among the 12 genotypes, several including TgCatBr10, 22, 23, 28, 31, 32, 37, TgCatBr15, and TgCatBr20 had the combination of type I/III alleles at different loci. Genotype of TgCatBr3, 4 had a combination of type II/III alleles. Two genotypes including TgCatBr1, 7 and TgCatBr5, 11, 16 had the combination of three alleles I/II/III and I/III/u-1, respectively. Six genotypes including TgCatBr2, 12, 17, 21, 30, TgCatBr9, 19, TgCatBr18, TgCatBr25, TgCatBr26 and TgCatBr34 had the combination of four alleles at different loci (I/II/III/u-1). TgCatBr3, 4 had type III alleles at all loci except a type II allele at c22-8 locus. TgCatBr15 had type III alleles at all loci except a type I allele at c29-2 locus. For both cases, the rare alleles were confirmed by replicate reactions of PCR-RFLP experiments. No archetypal genotype was found among these Brazilian cat isolates.

There were a few genotypes that differed at only one locus (Table 2). These included GUY-DOS and GUY-MAT differing at locus c29-2, TgCatBr10, 22, 23, 28, 31, 32, 37 and TgCatBr20 at locus L358, MAS and TgCatBr34 at locus BTUB, and TgCatBr9, 19 and TgCatBr26 at locus BTUB. All these differences were confirmed by replicate reactions of PCR-RFLP experiments.

Mixed genotypes were detected in isolate TgCatBr6. From gel image of marker *PK1* (Fig. 2), it was clear that TgCatBr6 had the DNA banding pattern comparable with a mixture of alleles I and u-1. The mixture of two alleles were also observed at loci c22-8, *BTUB*, *GRA6* and *SAG2* (gel images not shown). Fourteen clones obtained from TgCatBr6 by limiting dilution identified two genotypes, 13 of the 14 clones were identical to genotype of TgCatBr5. One clone designated as TgCatBr6-20 was a new genotype, which had a type I allele at *SAG2* locus and was identical to TgCatBr18 at the other eight loci. All 14 clones had a u-1 allele at *PK1* locus and no type I allele was identified, suggesting additional genotype(s) exist in the TgCatBr6 population. This conclusion is further supported by typing at *BTUB* locus, in which only type III allele was found in all 14 clones whereas mixing of I and III alleles were originally observed in TgCatBr6.

4. Discussion

A total of nine three-way PCR-RFLP markers were developed in this study. These markers had a similar resolution to multilocus DNA sequencing techniques in identifying and distinguishing non-archetypal isolates. The eight atypical isolates (seven atypical and one recombinant I/III type, see Table 2) previously studied by multilocus DNA sequencing were typed to individual level without ambiguity by the nine three-way markers, indicating their high discriminatory power. Even though all these markers were initially tested based on their polymorphisms among the three archetypal lineages, additional alleles were revealed. Both c22-8 and c29-2 were characterised by a fourth allele (u-1), and *PK1* was characterised by the fourth (u-1) and fifth (u-2) alleles

Table 2
Summary of multilocus genotyping

Strains	Previous typing	Host[a]	Locat.[b]	Markers									This study
				c22-8	c29-2	L358	PK1	SAG2	BTUB	GRA6	SAG3	Apico	
RH88	I	Hm	USA	I	I	I	I	I	I	I	I	I	I
GT1	I	Goat	USA	I	I	I	I	I	I	I	I	I	I
ENT	I	Hm	FR	I	I	I	I	I	I	I	I	I	I
VEL	I	Hm	USA	I	I	I	I	I	I	I	I	I	I
PTG	II	Shp	USA	II	II	II	II	II	II	II	II	II	II
DEG	II	Hm	FR	II	II	II	II	II	II	II	II	I	II
PIH	II	Hm	USA	II	II	II	II	II	II	II	II	II	II
CTG(CEP)	III	Cat	USA	III	III	III	III	III	III	III	III	III	III
STRL	III	Hm	USA	III	III	III	III	III	III	III	III	III	III
VEG	III	Hm	USA	III	III	III	III	III	III	III	III	III	III
CAST	Atypical	Hm	USA	II	I	III	I	I	I	I	I	III	Atypical
COUGAR	Atypical	Cgr	CA	II	u-1	I	u-2	II	II	II	III	I	Atypical
MAS	Atypical	Hm	FR	u-1	I	I	III	II	III	III	III	I	Atypical
CASTELLS	Atypical	Shp	URG	III	I	I	III	II	III	III	III	I	Atypical
VAND	Atypical	Hm	FrGy	III	I	III	III	II	III	III	I	I	Atypical
GUY-DOS	Atypical	Hm	FrGy	II	III	I	III	II	III	III	I	I	Atypical
GUY-MAT	Atypical	Hm	FrGy	II	I	I	III	II	III	III	I	I	Atypical
GPHT[c]	I/III	Hm	FR	u-1	I	I	I	I	I	II	III	I	Atypical
TgCatBr1 (2)[d]	I[e]	Cat	Brazil	I	III	I	II	II	III	III	III	III	Atypical
TgCatBr2 (5)[c]	I[e]	Cat	Brazil	u-1	I	I	I	I	I	II	III	I	Atypical
TgCatBr3 (2)	III[e]	Cat	Brazil	II	III	III	III	III	III	III	III	III	Atypical
TgCatBr5 (3)	III[e]	Cat	Brazil	I	I	I	u-1	III	III	III	III	I	Atypical
TgCatBr6	III[e]	Cat	Brazil	I/u-1	I	I	I/u-1	I/III	I/III	II/III	III	I	Atypical
TgCatBr9 (2)	I[e]	Cat	Brazil	I	I	I	u-1	I	III	II	III	I	Atypical
TgCatBr10 (7)	III[e]	Cat	Brazil	I	I	I	III	III	III	III	III	III	Atypical
TgCatBr15	III[e]	Cat	Brazil	III	I	III	III	III	III	III	III	III	Atypical
TgCatBr18	III[e]	Cat	Brazil	u-1	I	I	u-1	III	III	II	III	I	Atypical
TgCatBr20	III[e]	Cat	Brazil	I	I	III	III	III	III	III	III	III	Atypical
TgCatBr25	III[e]	Cat	Brazil	u-1	I	I	II	III	III	II	III	I	Atypical
TgCatBr26	I[e]	Cat	Brazil	I	I	I	u-1	I	I	II	III	I	Atypical
TgCatBr34	I[e]	Cat	Brazil	u-1	I	I	III	II	I	III	III	I	Atypical

[a] For Host, Hm, human; Shp, sheep; Cgr, Cougar.
[b] For location (Locat.), CA, Canada; FR, France; FrGy, French Guyana; URG, Uruguay.
[c] GPHT and TgCatBr2 are identical.
[d] The digit in parenthesis is the number of Brazil cat isolates belonging to the genotype. TgCatBr1 represents two isolates (TgCatBr1 and 7); TgCatBr2 represents five isolates (TgCatBr2, 12, 17, 21 and 30); TgCatBr3 represents two isolates (TgCatBr3 and 4); TgCatBr5 represents three isolates (TgCatBr5, 11 and 16); TgCatBr9 represents two isolates (TgCatBr9 and 19); TgCatBr10 represents seven isolates (TgCatBr10, 22, 23, 28, 31, 32 and 37).
[e] Typing of TgCatBr isolates was determined at *SAG2* locus by the method of Howe et al. (1997) and reported by Dubey et al. (2004).

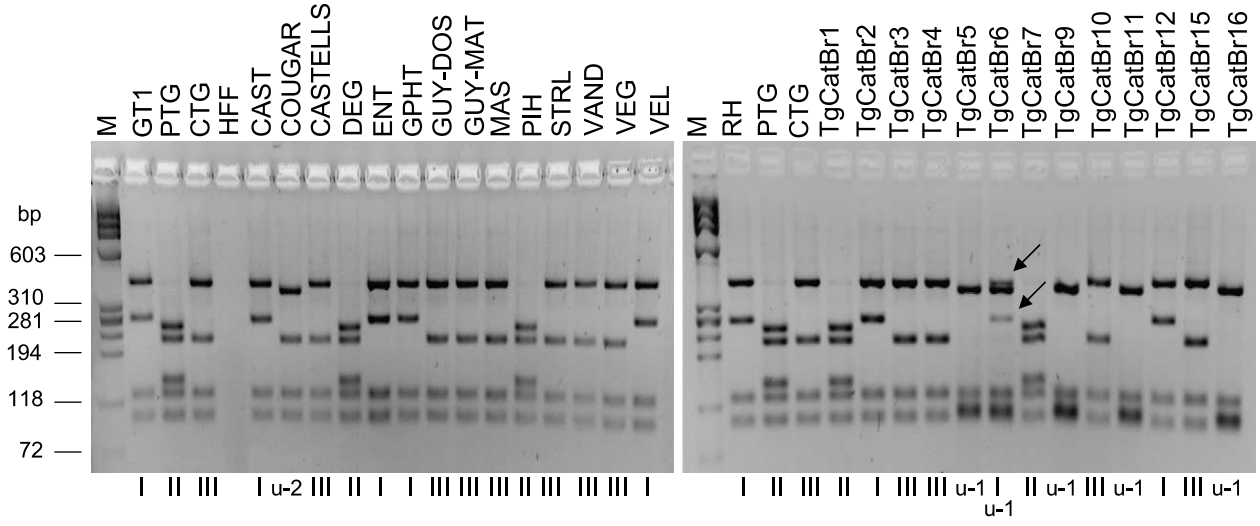

Fig. 2. Gel images for representative *Toxoplasma gondii* strains at locus *PK1*. Strain names are listed on top and the genotyping result is listed at bottom of the gel. Non-clonal allele u-1 was found in strains TgCatBr5, 9, 11 and 16. Allele u-2 was identified in COUGAR strain. Mixed types were found in sample TgCatBr6 and the arrows indicated the DNA bands represent type I allele. HFF: human foreskin fibroblast cell control. M: DNA molecular marker phiX174 DNA-*Hae* III digest.

(Table 2). Nineteen different genotypes occurred among the 36 atypical isolates of which five pairs only differed at one locus, including GUY-DOS vs. GUY-MAT at locus c29-2, TgCatBr15 vs. TgCatBr20 at locus c22-8, TgCatBr10 vs. TgCatBr20 at locus L358, TgCatBr9 vs. TgCatBr26 and MAS vs. TgCatBr34 at locus *BTUB*. In addition, TgCatBr3 and 4 had type III alleles at eight of the nine loci except the type II allele at c22-8 locus, and TgCatBr15 had type III alleles at eight of the nine loci except a type I allele at c29-2 locus. All these highlight the necessity of using multiple markers to achieve high resolution. However, this is limitation of the nine markers and they may not be able to distinguish closely related isolates within a clonal lineage. To overcome this limitation, microsatellite analysis may be used in combination with these RFLP markers. This approach should be facilitated by recently developed multiplex PCR of microsatellites for typing in *T. gondii* (Ajzenberg et al., 2005). As the rates of mutation of microsatellites (10^{-2}–10^{-5} per locus per replication) are much higher compared with that of point mutations in RFLP markers (10^{-9}–10^{-10}) (Goldstein and Schlotterer, 1999), microsatellites are especially useful in revealing very recent mutations in closely related isolates within a lineage, whereas the stable point mutations make RFLP markers the better choice for teasing apart more distantly related strains into different clonal branches. As multilocus PCR-RFLP genotyping is simple and cost-effective compared with DNA sequencing, it should prove attractive as a rapid and practical tool for identification and estimation of genetic diversity in *T. gondii*.

It is of great interest to link genotypes to phenotypes in a pathogen. Since the discovery of the strong link between type I *T. gondii* strain and acute virulence in mice over a decade ago (Sibley and Boothroyd, 1992), biological differences among different clonal types of *T. gondii* strains has been observed (Saeij et al., 2005). If genotypes are characterised by different phenotypes, then a parasite's biological characteristic can be predicted from its genotype. If a *T. gondii* population is clonal, then genotyping would require one or just a few genetic loci because of the strong linkage disequilibrium. However, even though *T. gondii* strains from human and domestic animals in North America and Europe are essentially clonal (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002), accumulating data from isolates sourced from wild animals or remote geographical regions suggests a highly diverse population in this parasite (Ajzenberg et al., 2004; Lehmann et al., 2004; Miller et al., 2004). Here, 12 genotypes were identified in the 28 Brazilian cat isolates and each genotype has a combination of 2 – 4 alleles, indicating their diversity. This may suggest a high rate of genetic exchange such as that observed in *T. gondii* chicken strains in Brazil (Lehmann et al., 2004). Taken together, these data suggest that *T. gondii* has an epidemic rather than clonal population structure (Tibayrenc and Ayala, 2002). Therefore, typing of *T. gondii* using a single marker is severely limited in its ability to detect non-clonal strains. Previous studies solely based on the *SAG2* locus for genotyping should be reconsidered in light of these new results. To more fully understand the phenotypic attributes of particular strains, we must identify *T. gondii* strains by high resolution markers. Assuming *T. gondii* genomes exchange randomly in the environment, the possibility of finding two different lineages sharing the same alleles at nine unlinked loci can be as low as 5×10^{-5} (1 in 3^9). In other words, an identical genotype at the nine unlinked loci should be a strong indicator of a clonal lineage. This level of confidence should prove suitable for studies on characterising associations between genotypes and phenotypes.

Mixed infection of *T. gondii* strains in intermediate hosts has been reported previously (Ajzenberg et al., 2002; Aspinall et al., 2003; Dubey et al., 2003, 2005; Pena et al., 2006). Detection of mixed infection is of particular interest in epidemiological studies. For genetic exchange to occur, the definitive host cats must ingest different types of parasites from their prey (intermediate hosts) at nearly the same time.

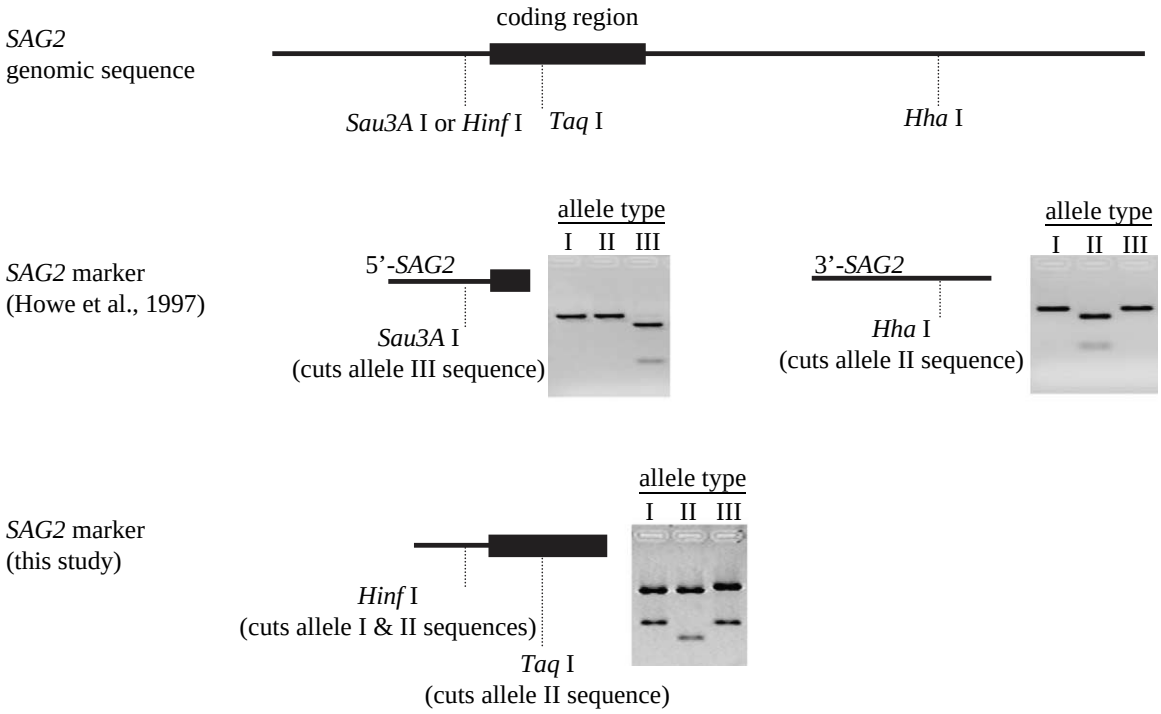

Fig. 3. Comparison of *SAG2* markers. Genomic DNA sequence is represented by the horizontal line, the coding region of *SAG2* is indicated by the filled box. Approximate positions of single nucleotide polymorphisms (SNPs) recognised by restriction enzymes are indicated by vertical dashed lines. Genotyping profiles of the three *Toxoplasma gondii* lineages (type I, II and III) are shown in the gel images. Genotyping at *SAG2* locus by the method of Howe et al. (1997) relies on the combined results from 5′-*SAG2* and 3′-*SAG2*. The newly developed *SAG2* marker in this study takes advantage of a sequence fragment that contains SNPs recognised by *Hinf* I as in 5′-*SAG2* and by *Taq* I in the adjacent coding region. This marker can differentiate all three alleles in one double-digestion reaction.

Examining mixed infection in cats would provide information on natural rates of recombination. In this study, one case of mixed infection was identified in 28 cat isolates, indicating it may not be a rare event for genetic recombination to occur in the definitive hosts. As 14 clones from isolates TgCatBr6 identified two genotypes but not all mixed alleles were found, additional clones are needed to reveal the complexity of TgCatBr6. Mixed infections in the prey may also provide an excellent opportunity for genetic crossing to occur in cats. The frequency of mixed infections in intermediate hosts will be a good indicator of the likelihood of the genetic exchange to occur in the field. Recently, a sensitive method has been developed to detect mixed genotypes in *T. gondii* samples at the *SAG2* locus (Sreekumar et al., 2005). The multilocus PCR-RFLP markers developed in this study have the advantage of checking multiple unlinked loci simultaneously and can be considered as another powerful assay added to the tool box for future studies.

The result of genotyping at *SAG2* locus for isolates TgCatBr1, 7 and 34 by the method of Howe et al. (1997) is different from that of *SAG2* in the current study (Table 2). We re-typed these isolates using both methods and ruled out possible errors in typing. A comparison of these two different *SAG2* markers is presented in Fig. 3. Howe's method relies on SNPs within both 5′ and 3′ non-coding regions of *SAG2* gene. For 5′-*SAG2* locus, restriction enzyme *Sau3A* I cuts the allele III sequence. The same restriction site is recognised by enzyme *Hinf* I that cuts the allele I and II sequences. For 3′-*SAG2* locus, enzyme *Hha* I cuts the allele II sequence. It is the combined results from both 5′-*SAG2* and 3′-*SAG2* that defines the archetypal I, II and III genotypes. However, the *SAG2* marker in this current study relies on a *Taq* I site in the upstream coding region of *SAG2* gene as well as the *Hinf* I site in 5′-*SAG2*. The *Taq* I site is shared by archetypal II and some atypical isolates such as TgCatBr1, 7 and 34, but the *Hha* I site in 3′-*SAG2* locus only exists in archetypal II isolates. This explains the discrepancy between the two different *SAG2* typing methods. From an evolutionary perspective, it is likely that the *Hha* I site is a 'younger' and archetypal II-specific allele.

As different laboratories use different markers for genotyping, it has been difficult to compare results. This study provides a set of PCR-RFLP markers that have high discriminatory power and are easy to use. Genotyping *T. gondii* isolates with these markers has proven useful. Adapting of these markers will facilitate epidemiological and population genetic studies in *T. gondii* in the future.

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