REVIEW ARTICLE

Moving towards an integrated approach to molecular detection and identification of *Toxoplasma gondii*

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(*Received 5 May 2009; revised 9 June and 5 July 2009; accepted 6 July 2009; first published online 21 September 2009*)

SUMMARY

The development of simple, sensitive and rapid methods for the detection and identification of *Toxoplasma gondii* is important for the diagnosis and epidemiological studies of the zoonotic disease toxoplasmosis. In the past 2 decades, molecular methods based on a variety of genetic markers have been developed, each with its advantages and limitations. The application of these methods has generated invaluable information to enhance our understanding of the epidemiology, population genetics and phylogeny of *T. gondii*. However, since most studies focused solely on the detection but not genetic characterization of *T. gondii*, the information obtained was limited. In this review, we discuss some widely used molecular methods and propose an integrated approach for the detection and identification of *T. gondii*, in order to generate maximum information for epidemiological, population and phylogenetic studies of this key pathogen.

Key words: *Toxoplasma gondii*, genetic characterization, molecular detection and identification.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular, apicomplexan parasite that infects all warm-blooded vertebrates, including mammals and birds. It is the only known species in the genus *Toxoplasma* and is considered to be one of the most successful eukaryotic pathogens in terms of the number of host species and percentage of animals infected worldwide. Up to one-third of the human population in the world is chronically infected (Dubey and Beattie, 1988; Tenter *et al*. 2000). Human infections are primarily caused by ingesting undercooked meat containing viable tissue cysts or by ingesting food or water contaminated with oocysts shed in the faeces from infected cats (Dubey, 2004). Primary infections in adults are mostly asymptomatic but lymphadenopathy or ocular toxoplasmosis can occur in some patients. Severe acute, disseminated toxoplasmosis can occur in immunocompetent human patients when infected with some isolates (Bossi and Bricaire, 2004). Infection acquired during pregnancy may spread and cause severe damage to the foetus. In immunocompromised patients, the reactivation of a latent infection can cause life-threatening encephalitis (Montoya and Liesenfeld, 2004).

T. gondii has subpopulation structures in different geographical regions. It has what appears to be a clonal population structure in North America and Europe, with 3 predominant lineages (named types I, II and III) defined by multi-locus enzyme electrophoresis (MLEE), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or microsatellite analysis (Darde *et al*. 1992; Howe and Sibley, 1995; Ajzenberg *et al*. 2002*a*). A recent report suggests the same clonal structure in Africa (Velmurugan *et al*. 2008). However, *T. gondii* isolates from animals and human patients in South America are diverse and largely distinct from those in North America and Europe (Ajzenberg *et al*. 2004; Khan *et al*. 2006; Lehmann *et al*. 2006; Pena *et al*. 2008; Dubey *et al*. 2008*d*). Historically, these distinct and non-type I, II or III parasites were designated as the 'atypical' or 'exotic' isolates. The low linkage disequilibrium among genetic loci of these isolates suggests that the parasites have undergone frequent sexual recombination (Ajzenberg *et al*. 2004; Lehmann *et al*. 2004; Su *et al*. 2006).

The consequences of infection with *T. gondii* depend on the host species and parasite genotypes.

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Parasitology (2010), **137**, 1–11.
doi:10.1017/S0031182009991065 Printed in the United Kingdom

In mice, type I lineages are uniformly lethal ($LD_{100}=1$); by contrast, the type II and III lineages are significantly less virulent ($LD_{100}>=10^3$) (Sibley and Boothroyd, 1992). In humans, disease manifestations vary widely, ranging from asymptomatic to severe acute toxoplasmosis (Bossi and Bricaire, 2004; Montoya and Liesenfeld, 2004). Type II is the predominant lineage causing human toxoplasmosis. However, there are biases between disease presentations and parasite genotypes. For examples, type I or type I-like atypical isolates are more likely to be involved in severe toxoplasmic retinochoroiditis in human patients (Grigg *et al*. 2001) and the atypical isolates often cause severe acute, disseminated toxoplasmosis in immunocompetent patients (Bossi and Bricaire, 2004).

Clinical symptoms of *T. gondii* infection are non-specific and unreliable for diagnosis. The conventional diagnosis of *T. gondii* infection usually employs serological tests, bioassays in cats and/or mice, or a combination of the 2 approaches (Dubey and Beattie, 1988). In the past 2 decades, the diagnosis of *T. gondii* infection by direct detection of parasite-specific DNA in biological samples using PCR-based molecular methods has gained popularity. The molecular diagnosis is more sensitive and cost-effective than the conventional methods (Schoondermark-Van De Ven *et al*. 1994; Bessières *et al*. 2009). There are hundreds of papers in current literature databases (such as PubMed) describing the use of these molecular methods. However, in this review article, rather than conduct an exhaustive review of all of the literature, we focus on key, representative articles to provide a perspective on establishing an integrated approach for the detection and characterization of *T. gondii*.

MOLECULAR METHODS FOR THE DETECTION AND IDENTIFICATION

Molecular methods rely on PCR for the specific detection or analysis of *T. gondii* DNA. These methods have proved to be simple, sensitive, reproducible and cost-effective, and have been applied to a variety of clinical samples from animals and humans (Bell and Ranford-Cartwright, 2002; Contini *et al*. 2005; Calderaro *et al*. 2006; Bastien *et al*. 2007). Molecular methods can be divided into 2 groups. The first group focuses on specific detection of *T. gondii* DNA in biological samples. The conventional PCR, nested PCR (n-PCR) and quantitative real-time PCR (qPCR) of repetitive DNA sequences belong to this group. The second group of molecular methods focuses on a high resolution identification of *T. gondii* isolates. The multilocus PCR-RFLP, microsatellite, and multilocus sequence typing (MLST) of single-copy DNA sequences belong to this group. In the following sections, we discuss the above methods in detail.

Molecular detection by conventional PCR, nested PCR (n-PCR) or quantitative real-time PCR (qPCR) of repetitive DNA sequences

To achieve high sensitivity, PCR of a small, repetitive DNA sequence is preferred, because the efficiency of amplifying a small DNA fragment is higher than that of a large one. In addition, there are more template copies in a repetitive sequence per organism. Three repetitive DNA sequences are often used for the detection of *T. gondii* in biological samples, including the 35-copy B1 gene, the 300-copy 529 bp repeat element and the 110-copy internal transcribed spacer (ITS-1) or 18S rDNA gene sequences. A molecular detection method (conventional PCR) for *T. gondii*, targeting the B1 gene, was first developed by Burg *et al*. (1989). This method has since been modified and adapted in different laboratories (Khalifa *et al*. 1994; Liesenfeld *et al*. 1994; Bretagne *et al*. 1995; Pelloux *et al*. 1998; Contini *et al*. 2002, 2005; Reischl *et al*. 2003; Switaj *et al*. 2005; Bastien *et al*. 2007). The 529 bp repeat element was identified by Homan *et al*. (2000), and it was reported to be 10- to 100-times more sensitive than the B1 gene (Homan *et al*. 2000; Reischl *et al*. 2003; Calderaro *et al*. 2006). The 110-copy ITS-1 or 18S rDNA has been used as the target in a few studies (Hurtado *et al*. 2001; Jauregui *et al*. 2001; Calderaro *et al*. 2006) and showed similar sensitivity to the B1 gene. To achieve higher sensitivity, n-PCR of B1 gene and ITS-1 sequences has been applied in some studies (Pelloux *et al*. 1998; Hurtado *et al*. 2001; Jauregui *et al*. 2001; Contini *et al*. 2002, 2005; Bastien *et al*. 2007), both markers showed high sensitivity, with the detection level being as low as 1 parasite (Hurtado *et al*. 2001; Jauregui *et al*. 2001; Calderaro *et al*. 2006). For a given repetitive sequence, n-PCR is more sensitive than conventional PCR. A case in point is that the n-PCR of the B1 gene is more sensitive than the conventional PCR for the detection of *T. gondii* in amniotic fluid samples from congenital toxoplasmosis of human (Okay *et al*. 2009). Recently, we employed a set of primers for n-PCR of *T. gondii* 18S rDNA, and found that it was more sensitive than the B1 gene (data not shown). The 18S rDNA marker is of particular interest, as it can distinguish several protozoan parasites which are closely related to *T. gondii*. There is further discussion of this marker in a later section (TOWARD AN INTEGRATED APPROACH FOR MOLECULAR DETECTION AND IDENTIFICATION) of this article.

In recent years, qPCR that targets the repetitive DNA sequences, has shown high sensitivity (detecting ~1 parasite genome equivalent) (Jauregui *et al*. 2001; Reischl *et al*. 2003; Contini *et al*. 2005; Calderaro *et al*. 2006; Edvinsson *et al*. 2006). The qPCR approach has gained popularity for not only detecting but also quantifying *T. gondii* in biological samples (Bell and Ranford-Cartwright, 2002; Contini

https://doi.org/10.1017/S0031182009991065 Published online by Cambridge University Press

et al. 2005). Its sensitivity is superior to n-PCR assays (Contini *et al*. 2005) and can detect *T. gondii* over a range of 6–7 orders of magnitude (Lin *et al*. 2000; Jauregui *et al*. 2001; Contini *et al*. 2005). Quantification of *T. gondii* by qPCR has been applied to the detection of the parasite in animal tissues and whole blood or amniotic fluids of human clinical samples (Lin *et al*. 2000; Jauregui *et al*. 2001; Kupferschmidt *et al*. 2001; Contini *et al*. 2005). Since it can estimate the intensity of *T. gondii* infection in the patients following drug treatment, qPCR is extremely useful to monitor the progression of infections under treatment and to diagnose low-level infections or carrier states (Contini *et al*. 2005). The 529 bp element is a preferred target for qPCR, as it provides a sensitivity of 6 repeat equivalents, or 1/50 of a genome equivalent (Kasper *et al*. 2009), currently representing the most sensitive assay for the detection of *T. gondii*.

Rapid and accurate detection of *T. gondii* infection is pivotal for prompt treatment of clinical toxoplasmosis. However, the above-mentioned detection methods (conventional PCR, n-PCR and qPCR of repetitive DNA sequences) provide no information beyond a positive diagnosis. To better understand the epidemiology of the parasite, there is an increasing interest and need in the genetic classification of *T. gondii*. In the following section, we discuss the methods used for the genetic identification and characterization of this parasite.

Molecular characterization by multilocus PCR-RFLP, microsatellite or multilocus sequence typing (MLST) analysis

For epidemiological studies, it is important to identify individual *T. gondii* isolates and to track the source of contamination. The commonly used methods for these purposes are multilocus PCR-RFLP, microsatellite or MLST typing. The PCR-RFLP is based on the ability of restriction endonucleases to recognize single nucleotide polymorphisms (SNPs), digest PCR products and subsequently display distinct DNA banding patterns on agarose gels by electrophoresis (Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Microsatellite analysis is based on the DNA sequence length polymorphisms of short nucleotide tandem repeats. The tandem repeats in *T. gondii* are often simple, consisting of as few as 2 nucleotides (di-nucleotide repeats), and occur 2–20 times (Blackston *et al*. 2001; Ajzenberg *et al*. 2002*a*, 2004). The MLST is based on DNA sequence polymorphisms including the SNPs, insertion and deletion of nucleotides in the sequence. SNPs and microsatellites are predicted to mutate at different evolutionary rates. For eukaryotes in general, the mutation rate for SNPs is estimated at 10^{-9}–10^{-10} per nucleotide position per replication, whereas the rate for microsatellites is 10^{-2}–10^{-5} per locus per replication (Goldstein and Schlotterer, 1999). Although the exact mutation rates of SNPs and microsatellites are not known in *T. gondii*, it is clear that the latter have a higher mutation rate and can provide enhanced resolution in typing (Ajzenberg *et al*. 2002*a*). The microsatellites are useful in distinguishing genetically closely related *T. gondii* isolates. Since multilocus PCR-RFLP and microsatellite typing are simple and cost effective compared with MLST typing, they are preferred tools for characterization of *T. gondii* in epidemiological studies.

A hallmark study of the epidemiology and population structure of *T. gondii* was conducted more than a decade ago on 106 isolates collected from humans and animals in North America and Europe (Howe and Sibley, 1995). In this study, 6 PCR-RFLP markers were used and 3 predominant lineages (types I, II and III) were identified. It was concluded that *T. gondii* had a clonal population structure (Howe and Sibley, 1995). Since then, different sets of multilocus PCR-RFLP or microsatellite markers have been applied to epidemiological and population studies (Blackston *et al*. 2001; Ajzenberg *et al*. 2002*a*, *b*, 2004, 2005; Lehmann *et al*. 2004, 2006; Khan *et al*. 2005*a*, *b*; Ferreira *et al*. 2006, 2008; Su *et al*. 2006). A key finding of these studies was that *T. gondii* isolates from South America were highly diverse and distinct from those of North America and Europe. It was concluded that, in South America, *T. gondii* has an epidemic population structure (Ajzenberg *et al*. 2004; Lehmann *et al*. 2004, 2006; Pena *et al*. 2008). However, the comparing of genotypes among different studies has been difficult or impossible, as different markers have been employed in different laboratories.

The disadvantage of using a single-copy gene or genomic sequence is that the sensitivity is compromised compared with a highly repetitive sequence. A technical challenge to multilocus typing is that often samples are limited both in volume and in DNA amount, particularly from tissues from clinical cases or the mouse bioassay. To alleviate this problem, we have developed a multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) method employing 10 genetic markers, including SAG1, SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico (Dubey *et al*. 2007*e*). The sensitivity of this method is estimated at 10 or more *T. gondii* genome equivalents, based on a previous study with 4 (SAG2, SAG3, BTUB and GRA6) of these 10 markers (Khan *et al*. 2005*a*). In contrast, the sensitivity of conventional PCR-RFLP is estimated at $\geqslant$100 *T. gondii* genome equivalents (unpublished data). In Mn-PCR-RFLP typing, all markers are pre-amplified by multiplex PCR using external primers in a single reaction, and the pre-amplified PCR products are used as templates to amplify each individual marker by n-PCR (Fig. 1). The advantage

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Table 1. Comparison of the methods for molecular detection and characterization of *Toxoplasma gondii*

Method	Sensitivity (no. of parasites/ per PCR reaction)	Advantage and limitation	Suitable application	References
qPCR of repetitive sequences	0·02 to 1 genome equivalent at 529-bp locus. ~1 genome equivalent at B1 locus	Highly sensitive in detection. Isolation of parasite not needed. Not suitable for typing.	Diagnosis	(Edvinsson *et al.* 2006; Kasper *et al.* 2009; Reischl *et al.* 2003) (Calderaro *et al.* 2006; Costa *et al.* 2000; Reischl *et al.* 2003)
n-PCR of repetitive sequences	1 to 5 genome equivalent at B1 locus	Sensitive. Isolation of parasite not needed. Not suitable for typing.	Diagnosis	(Calderaro *et al.* 2006; Contini *et al.* 2002)
Mn-PCR-RFLP	~10 genome equivalent	Less sensitive. High resolution in typing. Isolation of parasite may not be needed.	Epidemiology and population genetics	(Khan *et al.* 2005*a*)
Mn-PCR-based sequencing	~10 genome equivalent	Less sensitive, high resolution in typing. Isolation of parasite may not be needed.	Epidemiology, population genetics and phylogenetics	This study
Conventional PCR-based sequencing	100 genome equivalent	Insensitive, high resolution in typing. Isolation of parasite needed.	Epidemiology, population genetics and phylogenetics	This study
Multiplex PCR of microsatellite markers	Not known	High resolution in typing	Epidemiology, population genetics	(Ajzenberg *et al.* 2005)

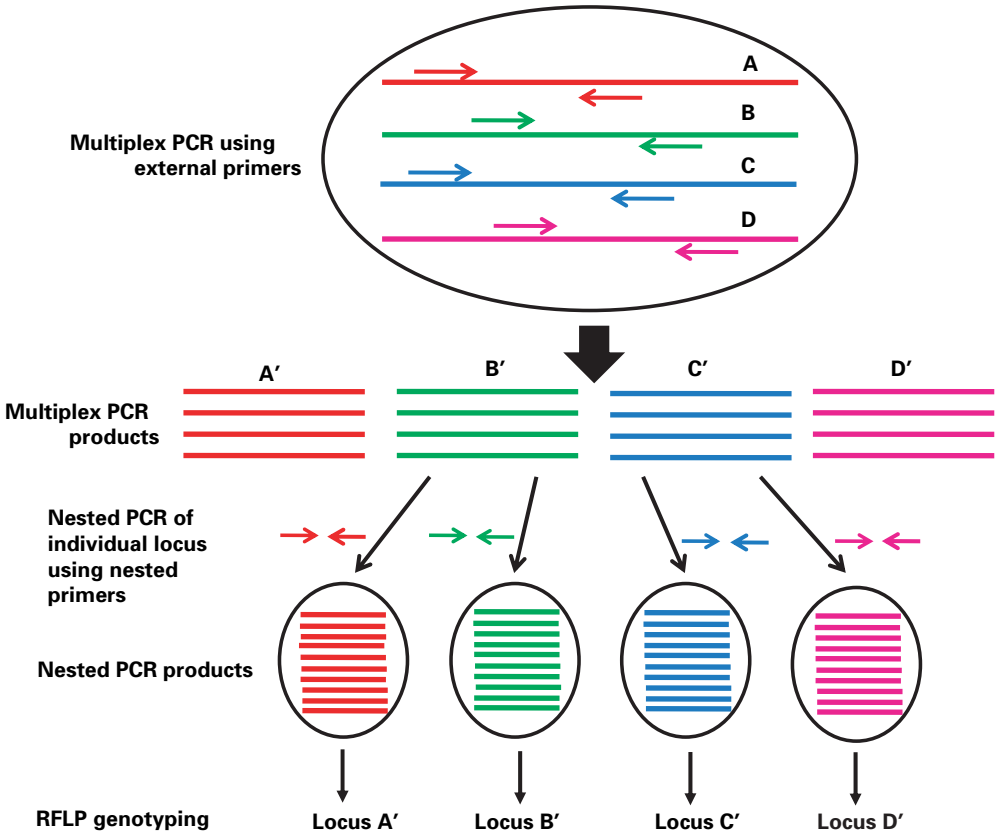

Fig. 1. Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing. Several genetic loci are pre-amplified by multiplex PCR with external primers. Then each individual locus is amplified by nested PCR using internal primers. The nested PCR products are treated with restriction enzymes and resolved in agarose gel to reveal strain-specific DNA banding patterns (RFLP typing). Horizontal lines represent DNA sequences and the horizontal arrows represent PCR primers.

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Table 2. Summary of primers for multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing (Su and Dubey, 2009)

(The multiplex PCR reaction is carried out in a vol. of 25 μl containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0·15 μM each of the external forward and reverse primers, 1 unit of FastStart DNA polymerase (Roche, Indianapolis, IN) and 1·5 μl of DNA samples. The reaction mixture is treated at 95 °C for 4 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 1 min and 72 °C for 2 min. Multiplex PCR amplified products are diluted (1 : 1) by adding 25 μl of nuclease-free water. The nested PCR reaction is carried out in a vol. of 25 μl containing 1 × PCR buffer, 2 mM $MgCl_2$, 200 μM each of the dNTPs, 0·30 μM each of internal forward and reverse primers, 1 unit FastStart DNA polymerase and 1·5 μl of diluted multiplex PCR products. The reaction mixture is treated at 95 °C for 4 min, followed by 35 cycles of 94 °C for 30 sec, 60 °C for 1 min and 72 °C for 1·5 min (for marker Apico, the annealing temperature is at 58 °C instead of 60 °C). For RFLP typing, 3–5 μl of nested PCR products are treated with restriction enzymes in a vol. of 20 μl and the digested samples are resolved in agarose gel to reveal DNA banding patterns (Su *et al*. 2006).)

Markers	Multiplex PCR primers (external primers)*	Nested PCR primers (internal primers)	Nested PCR (bp)	Restriction enzymes, NEB buffers, incubation temperature and time	Reference
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390	Sau96I + HaeII (double digest). NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Grigg *et al*. 2001)
5′-SAG2	Not needed. The DNA fragment for 5′-SAG2 is covered by the external primers of alt. SAG2.	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	242	MboI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Howe *et al*. 1997; Su *et al*. 2006)
3′-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222	HhaI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Howe *et al*. 1997)
alt. SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546	HinfI + TaqI, NEB3, BSA, 37 °C 30 min, 65 °C 30 min. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	225	NciI, NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Grigg *et al*. 2001)
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411	BsiEI + TaqI (double digest), NEB4, BSA, 60 °C 1 h. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	344	MseI, NEB2, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R:AGGTGCTTGGATATTCGC	521	BsmAI + MboII (double digest), NEB2, BSA, 37 °C 30 min, 55 °C 30min. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
C29-2	F: ACCCACTGAGCGAAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R:TGTCTAGGAAAGAGGCGC	446	HpyCH4IV + RsaI (double digest), NEB1, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418	HaeIII + NlaIII (double digest), NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
PK1	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	903	AvaI + RsaI (double digest), NEB4, BSA, 37 °C 1 h. 2·5% gel.	(Khan *et al*. 2005*b*; Su *et al*. 2006)
Apico	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640	AflII + DdeI (double digest), NEB2, BSA, 37 °C 1 h. 3% gel.	(Su *et al*. 2006)

* F, forward primer; R, reverse primer.

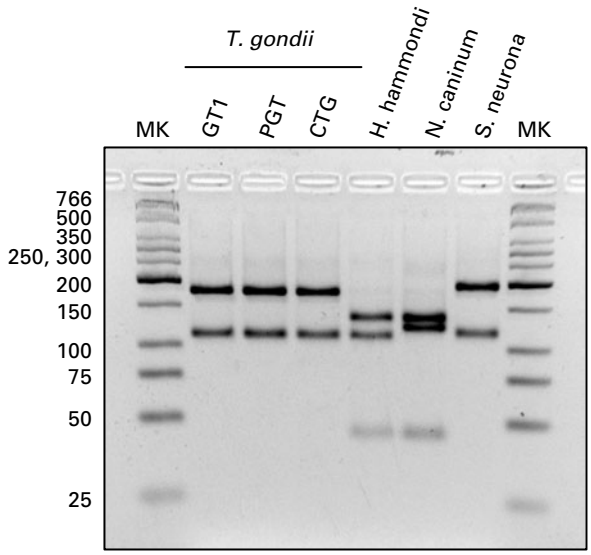

Fig. 2. PCR-RFLP gel image for *Toxoplasma gondii*, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis neurona* based on the 18S rDNA marker. PCR products from each parasite species were digested with 3 restriction enzymes *Dde*I, *Msp*I, *Hpy*188III (New England Biolabs Inc.) in a single reaction and DNA fragments were separated in 3% agarose gels. Samples GT1, PTG and CTG represent *T. gondii*. All 4 apicomplexan species have unique banding patterns and can be clearly distinguished (mouse and human DNA were used as negative controls and no PCR products were amplified, not shown). MK: Low molecular weight DNA ladder (New England Biolabs Inc.).

of this approach is that only a limited amount of individual sample is needed, which is particularly useful when only small amounts of 'precious' samples are available. Mn-PCR-RFLP analysis has been applied to the genetic typing of a large number of animal isolates bioassayed in mice, generating a substantial amount of data regarding genetic diversity and population structure of the parasite (Dubey *et al*. 2007*a-g*, 2008*a-e*; Pena *et al*. 2008; Sundar *et al*. 2008; Velmurugan *et al*. 2008; Dubey and Su, 2009). This method has also been used for the genetic characterization of *T. gondii* DNA extracted from the brains of sea otters (Sundar *et al*. 2008) and arctic foxes (Prestrud *et al*. 2008). Application of this method to a broad range of samples will greatly facilitate our understanding of molecular epidemiology and population diversity of *T. gondii* in the near future. When the amount of DNA for *T. gondii* isolates is not limited, the MLST typing is the method of choice because it has the highest resolution among all typing methods. Recent studies of *T. gondii* using this approach have revealed alleles unique to parasite isolates in Brazil (Khan *et al*. 2006, 2007), emphasizing the importance of a sequencing approach for studying the population genetics and molecular phylogeny of *T. gondii*.

TOWARD AN INTEGRATED APPROACH FOR MOLECULAR DETECTION AND IDENTIFICATION

Each of the detection and identification methods discussed above has its advantages and/or limitations, depending on application. A comparison of these methods is presented in Table 1. At present, most clinical studies have focused on detecting the presence of *T. gondii* DNA in biological samples using the highly sensitive nested or qPCR of repetitive DNA sequences, but little has been done to further characterize the parasites. This is because each of these markers (B1 gene, 529 bp repetitive element, or ITS-1) has identical or near-identical repetitive sequences, therefore they are not adequate for genetic typing. The conventional multilocus PCR-RFLP analysis method relies on single-copy polymorphic DNA sequences, and usually a relatively large amount of starting templates from the parasite is required. Therefore, it has been difficult to genetically identify *T. gondii* in clinical samples, due to the extremely small amounts of parasite DNA available. The recent development of the Mn-PCR-RFLP method makes it possible to genetically characterize or classify *T. gondii* from clinical samples with high resolution.

Here, we propose an integrated approach for the detection and genetic characterization of *T. gondii*. This approach should include detection of *T. gondii* infection in biological samples by n-PCR or qPCR of repetitive DNA sequences (employing the B1 gene or the 529-bp repeat element), followed by Mn-PCR-RFLP for the subsequent identification of *T. gondii*. There are a number of protocols developed in several laboratories for the detection of *T. gondii* by qPCR or n-PCR (Costa *et al*. 2000; Reischl *et al*. 2003; Edvinsson *et al*. 2006; Calderaro *et al*. 2006; Kasper *et al*. 2009). Although the sensitivity of these protocols varies among different laboratories, all studies demonstrate that the 529 bp repeat element is the most sensitive target, with a detection limit of $\geqslant 1/50$ of a genome equivalent (Kasper *et al*. 2009). Regardless of the protocol employed, all test-positive samples should be further characterized genetically by the Mn-PCR-RFLP typing method. The PCR primer sequences and experimental conditions for this typing method are listed in Table 2. This integrated approach will provide maximum information for epidemiological studies in order to address questions regarding the association of disease manifestations with parasite genotypes and virulence.

For phylogenetic studies, MLST is preferred for its high resolution. Conventional PCR based MLST requires the samples to have a relatively high concentration of genomic DNA ($\geqslant$100 genome equivalents per PCR). Taking the Mn-PCR approach, we have been able to generate quality DNA sequence data from samples containing as low as 10 genome

https://doi.org/10.1017/S0031182009991065 Published online by Cambridge University Press

Table 3. Summary of multilocus PCR-RFLP typing for *Toxoplasma gondii* reference isolates

	Genetic markers										
T. gondii reference isolates	SAG1*	(5′+3′) SAG2†	alt. SAG2‡	SAG3	BTUB	GRA6	C22-8	C29-2	L358	PK1	Apico
GT1, RH88 (type I)	I	I	I	I	I	I	I	I	I	I	I
PTG (type II)	II or III	II	II	II	II	II	II	II	II	II	II
CTG (type III)	II or III	III	III	III	III	III	III	III	III	III	III
TgCgCa1 (cougar, COUG)	I	II	II	III	II	II	II	u-1	I	u-2	I
MAS	u-1	I	II	III	III	III	u-1	I	I	III	I
TgCatBr5	I	III	III	III	III	III	I	I	I	u-1	I
TgCatBr64	I	I	u-1	III	III	III	u-1	I	III	III	I
TgRsCr1	u-1	I	II	III	I	III	u-2	I	I	III	I

* At SAG1 locus, type II and III are indistinguishable.
† *SAG2* marker based on 5′- and 3′-ends of the gene sequence (Howe *et al*. 1997).
‡ A *SAG2* marker based on the 5′-end of the gene sequence but different from 5′-SAG2 (Su *et al*. 2006).
u-1 and u-2 are new alleles that are different from the clonal type I, II and III alleles.

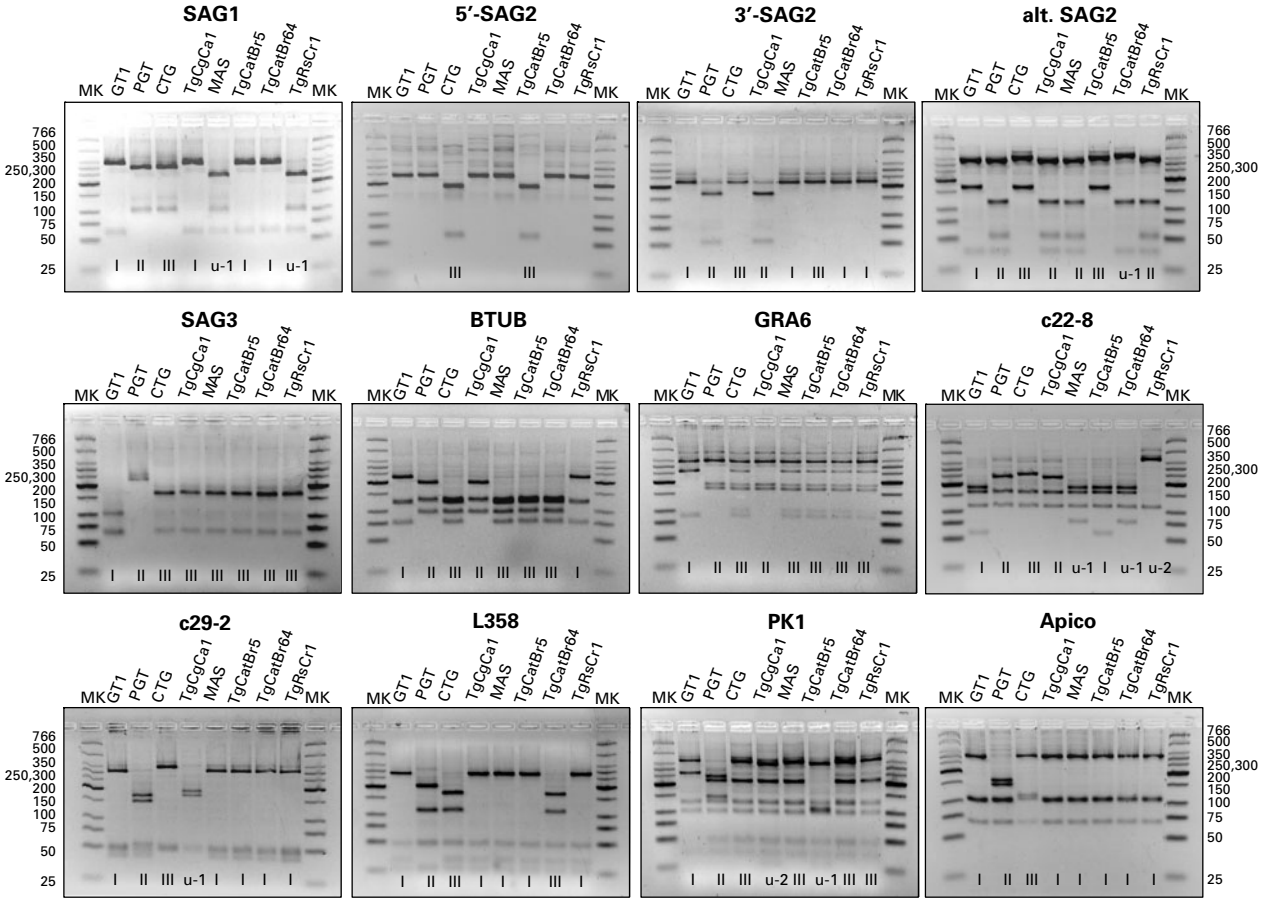

Fig. 3. Multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) analysis of *Toxoplasma gondii* samples using 10 different genetic markers. *T. gondii* reference strains are GT1 (or RH88), PTG, CTG, TgCgCa1 (a.k.a. Cougar, COUG), MAS, TgCatBr5, TgCatBr64 and TgRsCr1. Nested PCR products from each marker were digested with selected restriction enzymes (Table 2), and DNA fragments were separated in agarose. All markers were resolved in 2·5% gels, except that Apico was resolved in 3% gels. MK, low molecular weight DNA ladder (New England Biolabs Inc.).

equivalent (data not published). The major precaution for this approach is that, if errors occur in the early cycles of PCR, erroneous sequences may be generated. This problem can be solved by sequencing at least 2 independent PCR products from the same sample. To standardize studies and facilitate

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comparison among different laboratories, it is strongly recommended that all sequencing efforts include a few commonly used introns such as UPRT-1, UPRT-7, EF1 and HP (Khan *et al*. 2006, 2007) and the GRA6 gene (Fazaeli *et al*. 2000).

For genomic DNA samples extracted directly from animal tissues, we suggest screening all samples using n-PCR targeting the 18S rDNA sequence prior to Mn-PCR-RFLP typing for *T. gondii*. By sequence analysis of the 18S rDNA for a number of closely related apicomplexan parasites, we identified SNPs that can distinguish *T. gondii*, *Hammondia hammondi*, *Neospora caninum* and *Sarcocystis neurona* by RFLP analysis (Fig. 2). For n-PCR of 18S rDNA, the external primers are: Tg18s48F, CCA TGCATGTCTAAGTATAAGC and Tg18s359R, GTTACCCGTCACTGCCAC, which amplify a 312bp DNA fragment from *T. gondii*. The nested PCR primers are: Tg18s58F, CTAAGTATAAGC TTTTATACGGC and Tg18s348R, TGCCACG GTAGTCCAATAC, which amplify a 291bp DNA fragment from *T. gondii*. These primers were designed based on sequences conserved for the above 4 parasites. The GenBank Accession numbers of these sequences are M97703 (*T. gondii*), AF096498 (*H. hammondi*), U17346 (*N. caninum*) and U07812 (*S. neurona*). Using this marker, we recently detected a mixed infection of *Sarcocystis tenella* and *T. gondii* from sheep brain tissues (data not published). The 18S rDNA marker provides an excellent tool for detecting apicomplexans that are closely related to *T. gondii* in animal samples, which will add additional value to the studies on *T. gondii* infection in animal populations.

To monitor contamination of PCR amplification in Mn-PCR-RFLP genotyping, negative controls without DNA template should be included in each batch of experiments. In addition, a set of reference *T. gondii* isolates should be included to monitor the efficiency of PCR amplification and restriction enzyme digestion. This is very important, because for each batch of experiments there can be variation in efficiency of restriction digestion and sometimes incomplete digestion, and the concentration of electrophoretic gel can also vary slightly among experiments. Including *T. gondii* reference isolates in every experiment and on every electrophoretic gel will ensure the accuracy of results. The suggested references include RH88, GT1, PTG, CTG, TgCgCa1 (a.k.a. Cougar, COUG), MAS, TgCatBr5, TgCatBr64 and TgRsCr1 (Su *et al*. 2006; Dubey *et al*. 2009). The genetic data for these reference strains are summarized in Table 3. A set of gel images is presented in Fig. 3.

Currently, there is a lack of standard nomenclature for *T. gondii* genetic typing. Historically, genetic markers were selected based on polymorphisms in DNA or protein sequences among the clonal type I, II, and III lineages, and the corresponding alleles were designated either as I, II, III, or 1, 2, 3, respectively (Darde *et al*. 1992; Sibley and Boothroyd, 1992; Ajzenberg *et al*. 2004; Ferreira *et al*. 2006; Khan *et al*. 2006; Su *et al*. 2006; Pena *et al*. 2008). Alleles which are distinct from those of types I, II or III were designated as either unique-1 (u-1), unique-2 (u-2), unique-3 (u-3) etc., or 4, 5, 6, etc. In Table 3 and Fig. 3, we use the former scheme to describe new alleles.

In summary, the application of an integrated approach, combining molecular detection and high resolution genetic characterization, should assist in enhancing our understanding of the molecular epidemiology, population genetics and phylogeny of *T. gondii*, which will be beneficial to the control of *T. gondii* transmission and a reduction of toxoplasmosis in humans and animals.

ACKNOWLEDGEMENTS

This work was supported in part by the Department of Microbiology, University of Tennessee Knoxville (CS, Startup grant); the Chinese National Special Research Program for Non-Profit Trades (XQZ, Grant No. 200803017); and the Program for Changjiang Scholars and Innovative Research Team in University – China (XQZ, Grant No. IRT0723).

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https://doi.org/10.1017/S0031182009991065 Published online by Cambridge University Press

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