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Rapid genotyping of *Toxoplasma gondii* by pyrosequencing

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ABSTRACT

Most human infections with the protozoan parasite *Toxoplasma gondii* are asymptomatic, but severe symptoms can occur in immunocompromised patients, in developing foetuses, and in ocular infections in immunocompetent individuals. The majority of *T. gondii* strains can be divided into three main lineages, denoted types I, II and III, which are known to cause different clinical presentations. Simple molecular methods with the capacity to discriminate rapidly among strains may help to predict the course of infection and influence the choice of treatment. In the present study, real-time PCR followed by pyrosequencing was used to discriminate among types I, II and III by analysis of two single nucleotide polymorphisms in the *GRA6* gene. Twenty-one isolates of *T. gondii* characterised previously were analysed. Three different *GRA6* alleles detected by the pyrosequencing technique identified types I, II and III isolates correctly, while four atypical isolates possessed either the *GRA6* allele 1 or the *GRA6* allele 3. Reproducibility was 100%, and typeability, when including atypical strains, was 81%. It was also possible to discriminate a mixture of two genotypes. The method was used to identify *GRA6* type II in blood and lung tissue from an allogeneic transplant recipient with toxoplasmosis.

Keywords Genotyping, lineages, PCR, pyrosequencing, *Toxoplasma gondii*, typing

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INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite with worldwide distribution that commonly infects warm-blooded animals, including humans. Primary infections are usually asymptomatic, or are characterised by mild, flu-like symptoms. When host immunity is acquired, the parasite converts to a slow-dividing form, contained in intracellular cysts, and establishes chronic infection. In general, the consequences of toxoplasmosis can be severe in three clinical settings: reactivated infections in immunocompromised patients; congenital infections; and ocular infections.

Multilocus analysis has demonstrated that *T. gondii* has a population structure comprising, at least in Europe and North America, three major clonal lineages, denoted types I, II and III [1–3]. So-called atypical and recombinant strains exist, but appear to be more frequent in other areas of the world [4]. More than 50 different markers have been described for typing *T. gondii*, with the techniques used including multilocus enzyme electrophoresis, restriction fragment length polymorphism analysis, random amplified polymorphic DNA PCR, microsatellite analysis and DNA sequencing [4]. To a certain extent, the clinical consequences of toxoplasmosis seem to depend on the genotype of the infecting strain, in association with the strain's phenotype and the host's characteristics. Type II is responsible for most cases of human toxoplasmosis, although types I and III have also been detected [3,5–8]. Type I, or

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mixed genotypes I/III, has been identified in severe cases of ocular toxoplasmosis in Brazil and the USA [9,10]. However, more data concerning the relationship between the genotype and the clinical manifestations of toxoplasmosis should be collected, and would improve our understanding of the connection between clinical course, outcome and strain type. Furthermore, strain genotyping may become increasingly important in the developing field of *Toxoplasma* detection in food. Simple and rapid molecular techniques are required to discriminate between *T. gondii* strains.

In recent years, pyrosequencing has emerged as a suitable technique for molecular genotyping of various microbes by analysis of single nucleotide polymorphisms (SNPs) [11–14]. Pyrosequencing has been used previously to identify *T. gondii* strains by analysis of the *SAG2* gene, and to discriminate among types I, II, III and mixed genotypes in a sample [15]. A high degree of polymorphism has been detected in the *GRA6* gene of *T. gondii*, and this has been used to discriminate among types I, II and III and atypical strains by sequencing techniques [4,16,17]. The present study describes a rapid molecular approach, based on pyrosequencing of a fragment of the *GRA6* gene, and its application in the characterisation of *T. gondii* isolates and clinical samples.

MATERIALS AND METHODS

T. gondii isolates

The various laboratories of the 'French parasitologist network for *Toxoplasma* isolate collection' (BRC ToxoBS group) have collected a large series of *T. gondii* isolates from human patients in France. Twenty-one *T. gondii* isolates provided by the BRC ToxoBS group, characterised previously by multilocus analysis, based on microsatellite and isoenzyme markers, were used [18,19]: type I strains were RH, ENT, GIL, CT1 and ATIH; type II strains were S3K, Pru, JONES, S1K-C, CHAM, Man-Nja, SZY-C, GANGI, CHAMON and ELG; type III strains were C56 and NED; and atypical strains were TONT, P80, VAND and RUB. DNA extraction was performed using a QIAamp DNA mini Kit (Qiagen, Hilden, Germany) [20]. Extracted DNA was quantified and its quality was controlled using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) [21], with 80 fg of *T. gondii* DNA considered to equal one genome [22].

Patient samples

A female patient, aged 63 years, with chronic myelogenous leukaemia who subsequently relapsed after autologous transplantation, was treated with allogeneic stem-cell transplantation. The patient was seropositive for *T. gondii* before transplantation. Treatment with trimethoprim–sulphamethoxazole, a commonly used prophylactic agent against toxoplasmosis, was stopped because of skin rash. The patient reported headache, and later developed fever and symptoms of an upper respiratory tract infection, including cough. Magnetic resonance imaging of the brain revealed a focal lesion. Blood samples were taken on three consecutive days. The patient died on day 3, and lung tissue was obtained at autopsy.

Real-time PCR

Primers and probes (Table 1) for real-time PCR amplification of *T. gondii* DNA and an internal amplification control (IAC) were designed using Beacon designer v.4.0 (Premier Biosoft International, Palo Alto, CA, USA). Real-time PCR was performed using a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) with 20-μL sample volumes containing uracil–DNA glycosylase (LightCycler FastStart DNA master hybridisation probes; uracil–DNA glycosylase, heat-labile; Roche Diagnostics). Human blood cell DNA (1 μg) was added when *T. gondii* isolates were analysed. A 176-bp fragment of the *GRA6* gene of *T. gondii* was amplified using 500 nM primers, 125 nM *Taq*Man probe and 2.4 mM $MgCl_2$, with 2 min at room temperature, followed by 10 min at 95°C and 50 cycles of 95°C for 5 s and 65°C for 30 s.

The pcDNA3 plasmid (Invitrogen, Parsley, UK) was used as an IAC. A 265-bp fragment of the IAC was amplified using 125 nM primers (including the *Taq*Man probe) and the reaction conditions described above. The amount of pcDNA3 plasmid in a sample was balanced to a minimal detectable amount.

Standard curves were created to calculate amplification efficiency, error and the number of *T. gondii* genomes in unknown samples [21]. Amplification efficiency and error were determined for real-time amplification of the 176-bp fragment of the *GRA6* gene and the 265-bp fragment of the IAC.

Pyrosequencing

Discrimination of *T. gondii* types I, II and III was achieved by pyrosequencing analysis of two SNPs of the *GRA6* gene (accession numbers AF239283–AF239292). The variable nucleotides in the *GRA6* gene occur at positions 162 and 171 [16]. An

Table 1. Primers and *Taq*Man probes used in real-time PCRs to amplify and detect a 176-bp fragment of the *GRA6* gene of *Toxoplasma gondii*, and a 265-bp fragment of the pcDNA3 plasmid used as internal amplification control (IAC). A primer complementary to the *GRA6* fragment was used for pyrosequencing

Target	Forward primer 5′ → 3′	Reverse primer 5′ → 3′	Probe 5′ → 3′
Real-time PCR *GRA6*	Biotin-TCGTCAATTCGTTGGGTGGA	TGTATCATCTTCAGCTAACGAGTC	FAM-CACCGCCCATCGCCGAAGAGTTG-BHQ1
Real-time PCR IAC	TCGACTCTCAGTACAATCTG	ATGGGCTATGAACTAATGAC	HEX-TGCCGCATAGTTAAGCCAGTATCT-BHQ1
Pyrosequencing *GRA6*	–	CGCAGCAGACAGCG	–

11-base fragment of the *GRA6* gene (bases 162–172) was analysed by pyrosequencing. A specific nucleotide dispersion order was programmed according to the known *GRA6* sequences. Nucleotides were dispersed into the reaction mixture one at a time, so that incorporation of a complementary nucleotide resulted in a peak. A 'single peak' corresponds to a single base, and a 'double peak', which has greater amplitude than a 'single peak', corresponds to two consecutive identical bases. Pyrosequencing was performed using 0.35 μM pyrosequencing primer in an MA96Q pyrosequencing instrument (Biotage AB, Uppsala, Sweden) according to the instructions of the manufacturer.

Reproducibility (R) and typeability (T) of the pyrosequencing assay were determined by three independent analyses of the 21 *T. gondii* isolates. R was calculated as $R = N_r/N$, where N_r = the number of isolates assigned the same type on repeat testing, and N = the number of isolates tested. T, used as a measure of the ability of the pyrosequencing assay to discriminate between the different genotypes tested, was calculated as $T = N_t/N$, where N_t = the number of typeable strains, and N = the number of isolates tested.

The ability of the method to detect more than one type in a sample was also tested. The DNA concentrations in extracts of RH (type I), ELG (type II) and NED (type III) were determined by NanoDrop spectrophotometry, and DNA belonging to two genotypes was mixed in 1:1 ratios. A mixture of RH, ELG and NED DNA was also analysed to determine whether three different genotypes could be detected in a sample.

RESULTS

In-vitro performance of real-time PCR

An amplification efficiency of 2.0 is usually considered to be a theoretical optimum [22]. For amplification of *T. gondii* DNA, the efficiency was 2.0, with error 0.01. The corresponding figures for amplification of the IAC were 1.56 and 0.07. The sensitivity of the PCR was 100 *T. gondii* genome equivalents in a reaction volume when the IAC was included.

In-vitro performance of pyrosequencing

Pyrosequencing was successful with samples for which real-time PCR was successful (100 genome equivalents in a real-time PCR reaction volume). Analysis of amplified non-biotinylated IAC fragments alone did not result in pyrograms. The technique of pyrosequencing allowed detection of the three different alleles of *GRA6*, with R = 100% (N_r = 21 and N = 21) for three independent runs using 21 *T. gondii* isolates, and T = 81% (N_t = 17 and N = 21). Three different *GRA6* alleles were associated with types I, II and III (Fig. 1). Type I was identified by G at position 162 and A at position 171 (allele 1). The corresponding SNPs were G and G (allele 2) for type II, and A and A (allele 3) for type III. The four atypical isolates, as defined by multilocus analysis, possessed either allele 1 (VAND and RUB) or allele 3 (TONT and P80). As shown in Fig. 2, it was possible to detect a mixture of two genotypes in a sample, but three genotypes could not be distinguished.

Clinical application

Quantification of *T. gondii* DNA in the patient samples showed 3.7×10^3, 4.6×10^4 and 6.0×10^4 *T. gondii* genome equivalents/mL blood, respectively, on three consecutive days, and a large amount of *T. gondii* DNA was found in the lung tissue. Pyrosequencing analysis revealed *GRA6* allele 2 in both blood and lung tissue, suggesting infection by a type II *T. gondii* strain (Fig. 1d,e).

DISCUSSION

The present study illustrates the use of real-time PCR and pyrosequencing to discriminate between *T. gondii* isolates of types I, II and III, and also the clinical application of the method. In this context, real-time PCR can also be used to quantify and monitor the amount of *T. gondii* DNA in patient samples. This seems to have clinical relevance, as the allogeneic transplant recipient died after a tenfold increase of parasite DNA in the blood over the course of 3 days. Such cases highlight the need for timely diagnostic procedures and aggressive treatment of the infection following diagnosis. However, the extent to which the *T. gondii* genotype affected the course of the infection remains unclear, as host genetics, prophylactic regimens, and also the condition of the immune system, influence the outcome. Thus, *T. gondii* genotypes should be determined for more cases of toxoplasmosis in order to investigate the connection between genotype and the course and consequences of the disease.

In a pyrosequencing assay described previously, two sets of primers were used for multiplex amplification of *SAG2* DNA [15]. The pyrosequencing assay described in the present study required only one set of primers, and an IAC could be included. Furthermore, the use of real-time PCR decreased the analytical time significantly. However, the analytical sensitivity of real-time PCR targeting the *GRA6* gene, which occurs in only one copy per parasite, did not

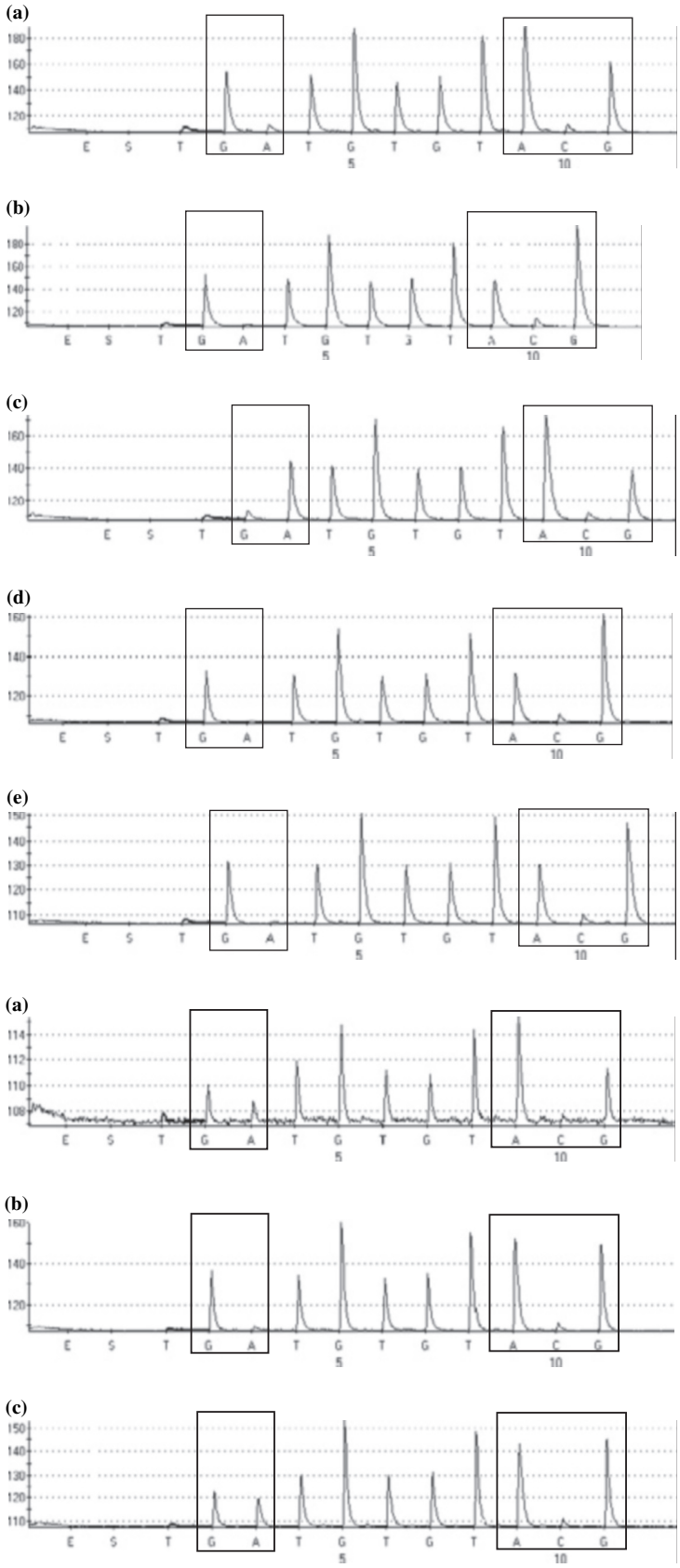

Fig. 1. Pyrosequencing analysis of the *GRA6* gene of *Toxoplasma gondii*. Pyrosequencing was performed by addition of enzyme (E), substrate (S) and a negative control (T). An 11-base fragment of the *GRA6* gene was sequenced; two single nucleotide polymorphisms (SNPs), enclosed in squares in the pyrograms and underlined in the following sequences, were analysed to discriminate types I, II and III. The sequence is: (a) GTGGTGTTAAG, for type I; (b) GTGGTGTTAGG, for type II; and (c) ATGGTGTTAAG, for type III. Blood samples (d) and lung tissue (e) from an immunocompromised transplant recipient with toxoplasmosis gave a type II isolate, shown by the sequence GTGGTGTTAGG.

Fig. 2. Pyrosequencing analysis of mixtures of *Toxoplasma gondii* types I, II and III. Discrimination of mixtures of two different *GRA6* alleles required analysis of the presence or absence of 'single peaks', and analysis of 'single peaks' with increasing amplitudes towards 'double peaks', or vice versa. A mixture of two genotypes is indicated by the presence of two different single nucleotide polymorphisms (SNPs), e.g., A and G, and is written as A/G. The SNPs analysed are enclosed in squares in the pyrograms, and are underlined in the following sequences: (a) G/ATGGTGTTAAG indicates a mixture of types I and III; (b) GTGGTGTTAA/GG indicates a mixture of types I and II; and (c) G/ATGGTGTTAA/GG indicates a mixture of types II and III.

match the sensitivity achieved in studies utilising repeated DNA targets, e.g., the 529-bp repeat fragment or the *B1* gene [7,21,22]. Consequently, genotyping may fail if a more sensitive PCR method has been used to detect the parasite and only a low amount of *T. gondii* DNA is present (personal unpublished observations).

Sequencing of the *GRA6* gene has revealed numerous SNPs [16]. The *GRA6* pyrosequencing assay presented here was designed to detect SNPs at only two sites, and therefore does not have the same typeability as would be achieved by sequencing longer fragments of the *GRA6* gene. However, it allowed correct identification of isolates belonging to the three main types of *T. gondii* that predominate in Europe. Atypical strains were identified as either type I or type III, and it was concluded that analysis of several independent genetic markers would be required if the aim was to identify atypical strains [4,23]. The analytical sensitivity of the assay might be increased by targeting repeated DNA elements. This has been done previously for PCR by analysis of the *B1* repeat element [24]. However, repetitive DNA elements are known to be highly polymorphic in sequence among repeat units, which may lead to confusing results.

Simultaneous detection of two different genotypes was possible, but challenging. As shown in Fig. 2, the absence or presence of a 'single peak' was indicative of type III, while discrimination of types I and II relies on analysis of peak heights. An SNP flanked by different bases might be preferred for this type of analysis, as this would result in the simple absence or presence of 'single peaks' when analysed by pyrosequencing, so that the results would be easier to interpret. It was not possible to detect three genotypes in a reaction. These difficulties have been described previously [15]. Nevertheless, the pyrosequencing assay provided a rapid and highly reproducible method to discriminate between *T. gondii* types I, II and III. Genotyping should be performed for more cases of toxoplasmosis to draw further conclusions concerning the influence of the precise *T. gondii* genotype on the course and outcome of the disease.

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