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An alternative nested-PCR assay for the detection of *Toxoplasma gondii* strains based on GRA7 gene sequences

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ABSTRACT

Toxoplasma gondii is a widespread parasite able to infect virtually any nucleated cells of warm-blooded hosts. In some cases, *T. gondii* detection using already developed PCR primers can be inefficient in routine laboratory tests, especially to detect atypical strains. Here we report a new nested-PCR protocol able to detect virtually all *T. gondii* isolates. Analyzing 685 sequences available in GenBank, we determine that *GRA7* is one of the most conserved genes of *T. gondii* genome. Based on an alignment of 85 *GRA7* sequences new primer sets that anneal in the highly conserved regions of this gene were designed. The new GRA7 nested-PCR assay providing sensitivity and specificity equal to or greater than the gold standard PCR assays for *T. gondii* detection, that amplify the B1 sequence or the repetitive 529 bp element.

1. Introduction

Toxoplasma gondii is an intracellular apicomplexan protozoon able to infect all warm-blooded vertebrates, including wild and farm animals. Infections in humans can evolve to a life-threatening disease in immunocompromised patients (Lewis et al., 2015). In pregnant women infection of the fetus can occur, potentially causing fetal death or injuries including ocular lesions, hearing deficits and neurological abnormalities (Delair et al., 2011; Stillwaggon et al., 2011; Wallon et al., 2004).

Early studies using isolates from Europe and North America classified *T. gondii* strains into three lineages designated Types I, II, and III, according to genetic characteristics and the level of virulence observed in a murine model (Dardé et al., 1992; Howe and Sibley, 1995). Subsequent studies have reported a wide genetic diversity of this parasite, especially in South American countries. In many cases these new isolates cannot be classified as one of the classical lineages, and it is not possible to establish a correlation between genotype and virulence (Rajendran et al., 2012; Carneiro et al., 2013; Cañón-Franco et al., 2014; Silva et al., 2014).

Since the introduction of molecular methods to detect *T. gondii* it has been noticed that low-level infections cannot be detected by serological tests (Chatterton et al., 2011). In some cases in which a rapid and efficient diagnose is determinant to guide the treatment (i.e congenital toxoplasmosis), the polymerase chain reaction (PCR) has been introduced as the most promising alternative (Bastien, 2002; Robert-Gangneux and Dardé, 2012). In the past few decades, different primer sets have been developed to detect *T. gondii.* In 1989, Burg et al., introduced the B1 gene as an efficient PCR target, since this sequence presents 35 repeats in the *T. gondii* genome. Since then, the B1 amplification has been used as a gold standard method for *T. gondii* detection, although it has shown low specificity in some cases (Kompalic-Cristo et al., 2004). A new promising marker consisting of a 529 bp fragment (RE) repeated 200–300 fold in the genome of *T. gondii* was described by Homan et al., in 2000. Several works have demonstrated that PCR reactions using the RE marker are at least 10 times more sensitive than using B1 (Cassaing et al., 2006; Fallahi et al., 2014; Reischl et al., 2003). Even though the 529 bp repetitive element showed greater sensitivity,

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0001-706X/

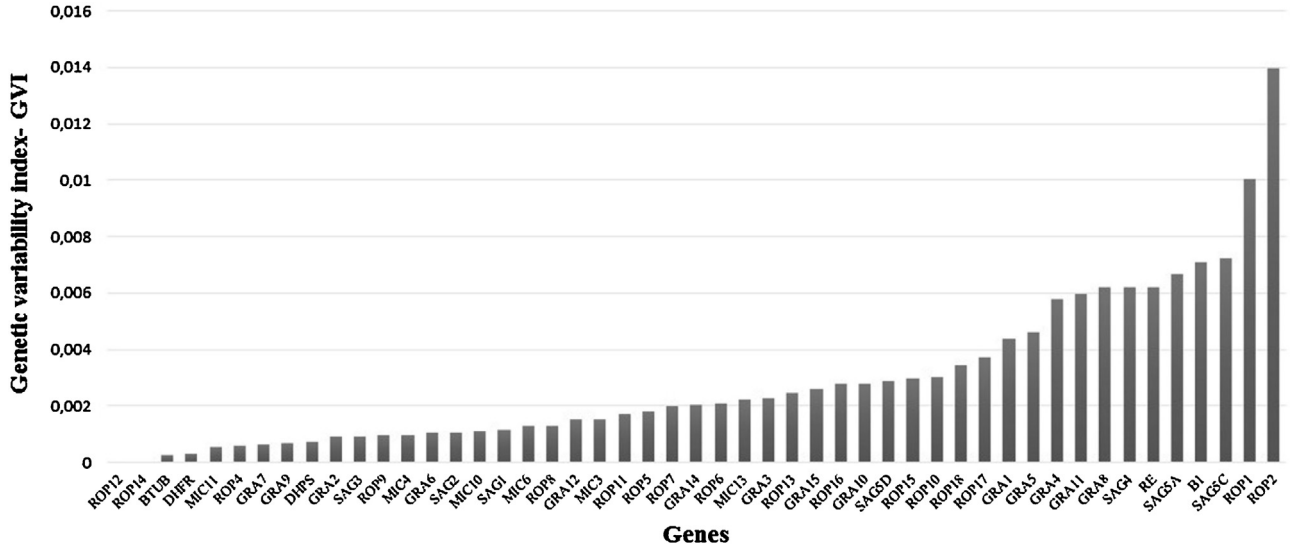

Fig. 1. Genetic variability of selected *T. gondii* genes. Selected genes were ranked from smallest to largest genetic variability, according to their respective genetic variability index (GVI).

the possibility that some strains have partially or entirely lost the repetitive units was reported, which can compromise the test efficiency (Wahab et al., 2010).

Since the available primers may not be effective for the detection of some variants of the parasite, the main objective of this work was to develop a new nested-PCR assay able to detect the clonal and atypical strains of *T. gondii*. To reach this objective, we analyzed a set of 685 sequences of different *T. gondii* strains and elected the *GRA7* gene as the best target for primer design. The validation of the new GRA7 nested-PCR assay comparing it with the two most widely used protocols indicates that our new method is a promising solution to detect a wide range of *T. gondii* strains.

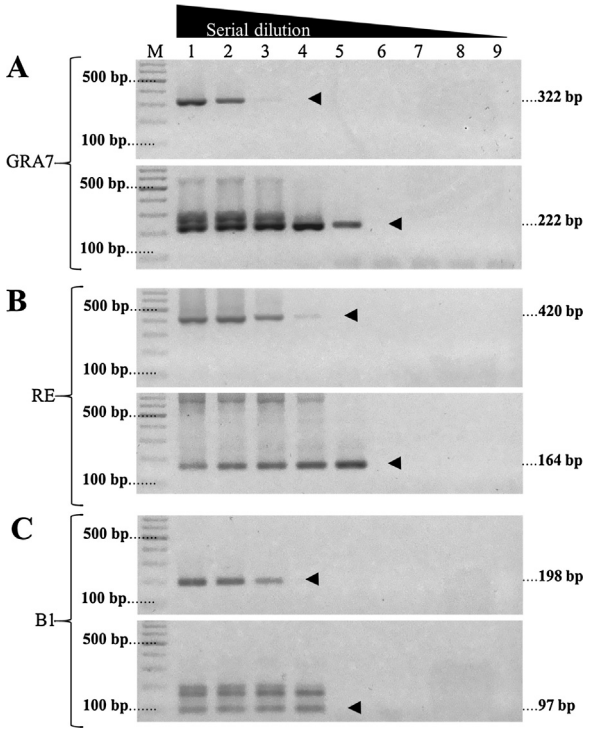

Fig. 2. Sensitivity test. A 40-fold serial dilution (Lanes 1–9) using total DNA extracted from infected RAW 264.7 cells as template was performed to determine the sensitivity of the (A) nested-PCR assay using *GRA7* primers; (B) nested-PCR assay using RE primers; and (C) nested-PCR assay using B1 primers. Within each panel, gel pictures above correspond to the first step and those below to the second step PCR. Black arrows indicate the expected amplicons of each reaction and its corresponding sizes are shown at right. M, 100 bp molecular-weight marker.

2. Materials and methods

Each set of DNA sequences were retrieved from GenBank (Benson et al., 2005), aligned using Muscle algorithm (Edgar, 2004) with default parameters (gap open penalty—12.0) and subsequently analyzed using MEGA software (Tamura et al., 2013) to determine the number of sequences in each alignment, the length of the sequences, and the total number of polymorphisms of each coding region. Primers were designed using primer-BLAST (Ye et al., 2012) and checked using AutoDimer (Vallone and Butler, 2004). *T. gondii* isolates TgCkBrRN2 (Ck2), TgCkBrRN3 (Ck3) and TgPgBrRN1 (Pg1) were recovered as previously described (Clementino Andrade et al., 2013). The isolates were maintained inoculating 150 cysts (Ck2, Ck3, Pg1, ME49,VEG) or 10^5 tachyzoites (RH) intraperitoneally into C57BL/6 or Swiss mice (n = 3). After three days, the mice were euthanized, and the tachyzoites of the isolates RH, Ck2 and Pg1 were recovered from the peritoneal exudate and seeded in RAW 264.7 cells. The isolates ME49, VEG and Ck3 were obtained directly from macerates of Swiss mice brains, thirty days after the infection. The DNA template were extracted using the QIAamp DNA mini kit (QUIAGEN Inc., USA), and quantified using SpectraMax 190 Microplate Reader (Molecular Devices). The two steps of nested-PCR assays were performed in 20 μL reactions containing 1.5 μL of template DNA, 0.25 μM of each primer, 1.5 mM $MgCl_2$, 0.01 U Taq DNA pol (Ludwig) and 0.2 mM of each dNTP. The first step PCRs were performed using the primer pairs GRA7FE/GRA7RE, NF1/NR1 and Oligo1/Oligo4 and nested-PCRs using GRA7FI/GRA7RI, NF2/NR2, and Oligo2/Oligo3 respectively. PCR products were resolved in a 1.5% agarose gel stained with ethidium bromide. Each reaction was done in at least three independent replicates. Primer sequences and thermocycling parameters are detailed in Tables 1 and 2 respectively. Serial dilution of the DNA template was prepared starting from a sample containing 206.6 ng/μL of total DNA from a RAW 264.7 culture infected with RH strain. The coverage tests were performed using DNA template extracted from infected RAW 264.7 cultures (RH, Ck2 and Pg1) or Swiss mice brains (ME49, VEG, Ck3).