

Comparison between two Real-time PCR assays and a nested-PCR for the detection of *Toxoplasma gondii*

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Abstract. *Background and aim of the work.* In recent years, the diagnosis of toxoplasmosis has been improved by Real-time PCR assays. In this study we compared the performances of two Real-time PCRs (FRET and TaqMan protocols) already described in the literature, and one nested-PCR, currently used in our laboratory for the molecular diagnosis of toxoplasmosis. *Methods.* We evaluated the sensitivity and the specificity of a FRET- and a TaqMan-based Real-time PCRs targeting a 529 bp repeat region and the 18S RNA gene, respectively, and a nested-PCR, targeting the B1-gene of *Toxoplasma gondii*. We also tested, through nested-PCR, 46 biological samples obtained during a period of 29 months from pregnant women or immunocompromised patients with suspected *T. gondii* infection. *Results.* The analytical sensitivity of nested and TaqMan PCRs was approximately 10^3 tachyzoites/ml. FRET assay showed a sensitivity of 10^2 tachyzoites/ml. Three out of 46 biological samples were nested-PCR-positive and these results were also confirmed by both Real-time PCRs. *Conclusions.* Nested- and real-time PCRs evaluated in this study resulted very sensitive and specific; in particular FRET PCR resulted more sensitive than the other assays, probably because of the greater copy number of the target sequence. Real-time PCR assays are easy-to-use, producing results faster than conventional PCR systems and reducing contamination risks. (www.actabiomedica.it)

Key words: *Toxoplasma gondii*, Real-time PCRs, Nested-PCR

Introduction

Toxoplasmosis is a worldwide disease caused by a protozoan parasite, *Toxoplasma gondii* (1). Although infections with this parasite are typically asymptomatic in healthy individuals, *T. gondii* causes substantial morbidity and mortality in immunocompromised patients (mainly AIDS patients and bone marrow and organ transplant recipients) (2, 3) and in congenitally infected infants (4).

A rapid, accurate and effective diagnosis is crucial and desirable to initiate adequate treatment in these two populations. Current diagnosis of toxoplasmosis relies either on serological detection of specific anti-*Toxoplasma* immunoglobulin, on culture of amniotic fluid or foetal blood, or on other non-specific indicators of infection (1). Serological testing may fail du-

ring the active phase of *T. gondii* infection because the antibodies titres are low; therefore, the high risk of congenital toxoplasmosis of a foetus may be undetected because the pregnant mother might test negative during the active phase of *T. gondii* infection. Moreover, serological tests may fail to detect *T. gondii* infection in certain immunocompromised patients due to the fact that the titres of specific antibodies anti-*Toxoplasma* may fail to rise in this type of patient. This is why the direct demonstration of the parasite in tissues or other biological fluids by PCR is a major breakthrough for the diagnosis of toxoplasmosis in these patients (5, 6). Mouse inoculation or tissue cultures of the clinical samples may confirm the infection by parasites but they are still impractical, labor-intensive and require several days to obtain results (7, 8). In the past decade, the use of PCRs has improved the dia-

gnosis of toxoplasmosis (9, 10). In particular, the newly developed Real-time PCR assays, allowing amplification and simultaneous PCR product detection, have accelerated the revelation of *T. gondii* DNA (from 24–48 h to less than 4 h) and minimised the risks of contamination that could be associated with conventional PCR. However, different factors might influence the PCR outcome: in particular, the choice of the DNA target and primers are generally considered as essential. At present, few DNA target loci have been described for *Toxoplasma* PCR, but different primer pairs have been used in many assays (11). The principal gene target remains the 35-fold repetitive and conserved B1 gene (12).

During 2005 we tested in our laboratory 6,398 serum samples belonging to 3,997 subjects (1,329 were pregnant women, 173 were immunocompromised patients, 42 were newborn and the others belonged to healthy individuals or subjects with pathologies other than toxoplasmosis) with an overall prevalence of *T. gondii* infection of 31.57%. In the light of our data we focused our attention on the diagnosis of toxoplasmosis in immunocompromised patients and in congenital toxoplasmosis since serological diagnosis in these cases could fail or could be difficult to interpret.

The aim of this study was to do a comparison between the analytical (specificity and sensitivity) and technical performances of two Real-time PCRs and a nested-PCR for the molecular diagnosis of *Toxoplasma gondii* infection, in order to select a molecular method able to overcome the limit of serological test to diagnose toxoplasmosis in immunocompromised patients and congenital toxoplasmosis. We also evaluated the economic aspects of the possible application of these three PCR assays in diagnostic laboratories.

Materials and methods

In order to evaluate the sensitivity of Real-time PCRs (13, 14) and nested-PCR (Amplimedical S.p.A., Italy) (12), to mimic conditions of clinical laboratory practice and to verify the possible effect of the host DNA upon the performances of the methods, *T. gondii* tachyzoites (RH strain), grown onto VERO cell BS CL 86 (African Green Vervet Monkey Kid-

ney, Istituto Zooprofilattico, Brescia, Italy) and counted by microscopy, were diluted both in culture medium and in blood (peripheral blood and cord blood from donors) *Toxoplasma*-negative samples (from 10^7 – 10^6 to 1 parasite/ml). DNA was extracted from 200 μ l of each of the prepared samples and submitted to PCRs as subsequently described. The analytical sensitivity was expressed as number of tachyzoites per ml and then converted in femtograms (fg) of *T. gondii* DNA revealed per reaction, considering that one *T. gondii* genome equals to about 100 fg and that we used an aliquot of 5 μ l for each PCR reaction.

The specificity of the Real-time PCRs was evaluated by testing DNA preparations from samples containing parasites other than *T. gondii* belonging as this latter to the phylum *Apicomplexa* (*Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* infecting blood and *Cryptosporidium* spp. infecting the small intestine), and *Leishmania infantum* that could be found in blood samples as *T. gondii*. Finally, we also tested DNA from VERO cells and culture medium. Moreover, negative controls for specimen processing and amplification (double-distilled water instead of DNA) were performed in each run of Real-time and nested-PCRs.

Three out of 46 biological samples (16 amniotic fluids, 13 blood samples, 7 cerebrospinal fluids, 7 tissue biopsies, 2 vitreous fluids and 1 lymph node biopsy) obtained during a period of 29 months from pregnant women or immunocompromised patients with suspected *T. gondii* infection, were nested-PCR-positive for *T. gondii* DNA. These samples, 2 cerebrospinal fluids (CSF) collected with an interval of one month between each other and 1 cerebral biopsy belonging to an immunocompromised patient (*T. gondii* IgG-positive and IgM-negative) admitted to our hospital with encephalitis, were also analysed by Real-time PCRs.

The extraction of DNA was performed from 200 μ l of each tested sample by High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) according to the manufacturer's specifications. An aliquot (5 μ l) of DNA was used for each PCR mixture.

Nested-PCR (12) was carried out in the GeneAmp® PCR System 9700 (Applied Biosystems) following the manufacturer's instructions. Amplification products were revealed by 4% agarose gel electrophoresis and ethidium bromide staining.

FRET (Fluorescence Resonance Energy Transfer) PCR, targeting *T. gondii* 529 bp repeated region (more than 300-fold) (13, 14), and TaqMan PCR, targeting *T. gondii* repetitive 18S rDNA (14), were performed as previously described (7, 15), with some modifications. Briefly, we used the LightCycler Fast-StartDNA Master^{PLUS} Hybridization Probes[®] Kit (Roche Diagnostics) for FRET PCR and the TaqMan[®] Universal PCR Master Mix (Applied Biosystems) for TaqMan PCR. Moreover, in the latter, for Uracil-N-Glycosylase (UNG) activation, we introduced an initial incubation for 2 min at 50°C and the products were analysed on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). For all PCR assays we used 5 µl of DNA for each reaction mixture. All samples were tested in duplicate in TaqMan assay and in single in the other two PCRs and, for some experimentally seeded samples (culture medium and blood samples), additional replicate experiments were performed to precisely determine the detection limits of the methods. Extreme physical separation and decontamination procedures (16) were used to avoid contamination by amplicons.

To control the course of extraction and check for PCR inhibitors, a β-actin gene (Applied Biosystems) was amplified by a TaqMan-based Real-time PCR at the same reaction conditions used for the 18S rDNA



Figure 1. Lane 1: molecular weight marker (1,114-19 bp); lane 2: Extraction negative control (double distilled sterile water); lane 3: DNA extracted from MEM medium without *Toxoplasma gondii*; lane 4: DNA extracted from peripheral blood without *Toxoplasma gondii*; lane 5 to 12: *Toxoplasma gondii* DNA from pure culture in MEM medium ten-fold diluted from 1.2×10^7 tachyzoites/ml to 1.2 tachyzoites/ml; lane 13: Extraction negative control (double distilled sterile water); lane 14 to 19: *Toxoplasma gondii* DNA from peripheral blood ten-fold diluted from 1.2×10^7 tachyzoites/ml to 12 tachyzoites/ml

T. gondii TaqMan-based Real-time PCR: a 2 min at 50°C for UNG activation, a 10 min hot-start at 95°C followed by 50 cycles of 15 s at 95°C and a combined annealing and extension step for 1 min at 60°C.

Results

The analytical sensitivity of nested and TaqMan PCRs in culture medium and in blood samples (peripheral and cord blood) was approximately 10^3 tachyzoites/ml (500 fg of DNA/reaction) (Figure 1 and 2A). Mean Ct (Cycle threshold) values of TaqMan as-

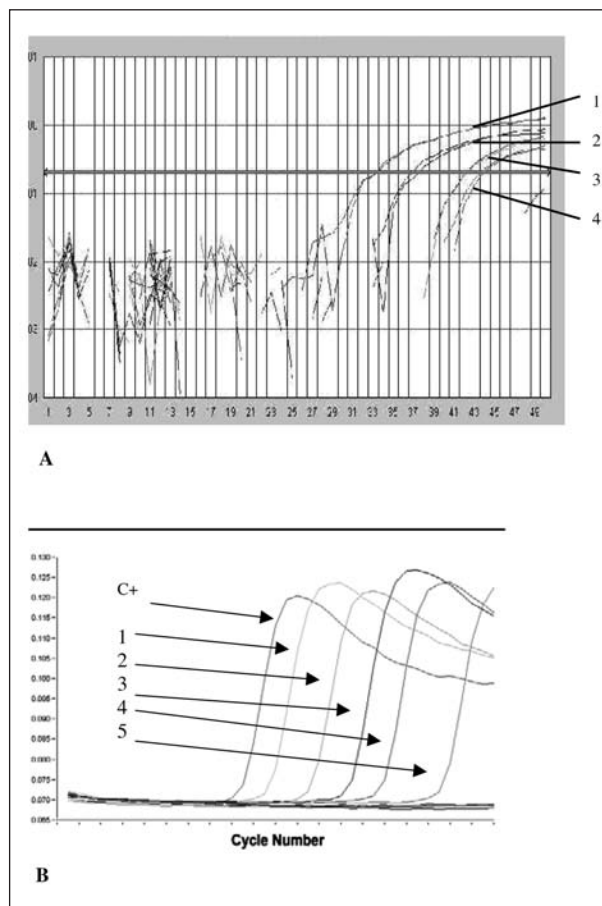


Figure 2. Sensitivity of the *Toxoplasma gondii* TaqMan and FRET Real-time PCRs. Amplification plots obtained for peripheral blood samples experimentally seeded with 10^6 (1), 10^5 (2), 10^4 (3), 10^3 (4) and 10^2 (5) *Toxoplasma gondii* tachyzoites per ml, by TaqMan (A) and FRET Real-time PCR (B). C+: positive control = *T. gondii* DNA

say ranged from 33.26 to 43.75 for peripheral blood and from 30.01 to 41.47 for cord blood. The assay was linear for a 10,000-fold range of tachyzoite concentrations in both biological specimens ($R^2=0.9638$ and $R^2=0.9845$, respectively) and a significant correlation coefficient ($r=-0.9817$ and $r=-0.9922$, respectively) between the number of tachyzoites and the mean Ct values was observed. The analytical sensitivity of nested-PCR in amniotic fluid and lymph node aspirate was 10-fold better than that observed in blood samples. Whilst TaqMan sensitivity was the same for amniotic fluid, lymph node aspirate and blood samples.

FRET assay showed a sensitivity of about 10^2 tachyzoites/ml (50 fg of DNA/reaction) in culture medium and in peripheral and cord blood (Figure 2B). Cp (Crossing point) values ranged from 18.84 to 34.35 for donor blood and from 20.29 to 34.31 for cord blood samples. The detection response was linear over a 100,000-fold range of tachyzoite concentrations for both biological samples ($R^2=0.9917$ and $R^2=0.9957$, respectively) and, also in this case, a significant coefficient of correlation ($r=-0.9958$ and $r=-0.9978$, respectively) between concentration of tachyzoites and Cp values was observed. The analytical sensitivity of FRET assay in amniotic fluid and lymph node aspirate was 100-fold better than that observed in blood samples.

No signal was detected by Real-time PCRs when DNA obtained from non-*Toxoplasma* protozoa species (*P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*, *L. infantum*, *Cryptosporidium* spp.), VERO cells and culture medium was used. Moreover, no amplification product was revealed from negative reaction controls.

With regards to clinical specimens, all three nested-PCR-positive samples were positive by FRET PCR (Cp=25.73 for cerebral biopsy, Cp=25.58 for the

first cerebrospinal fluid and Cp=32.85 for the second cerebrospinal fluid sample). The cerebral biopsy and the first cerebrospinal fluid sample also resulted positive by TaqMan assay; the second cerebrospinal fluid sample was negative. This sample was twice amplified in duplicate by TaqMan assay confirming the negative result (Table 1).

Analysis of clinical and experimentally seeded samples proved that they were free of PCR inhibitors since β -actin gene was successfully amplified and the spiked control was correctly amplified each time.

Conclusions

As for all parasitic diseases, the PCR diagnosis of toxoplasmosis is not standardized. It seems highly probable that there will be a consensus on Real-time PCR assays in the next few years (14). However, the consensus on the best sequence to be amplified will be more difficult. In this study we compared the performances of three PCR assays, targeting three different *T. gondii* genes (a commercial nested-PCR and two home-made Real-time PCRs).

All the PCR assays evaluated in the present study resulted very sensitive: considering that one *T. gondii* genome equals to about 100 fg (14, 17), the detection limit in blood was equivalent to approximately 500 fg of *T. gondii* genomic DNA for nested and TaqMan PCRs and 50 fg for FRET-based assay. This level of analytical sensitivity was in agreement with previous reports (14, 15).

We also evaluated Real-time PCR sensitivity using *T. gondii* tachyzoites diluted in other biological samples (amniotic fluid and lymph node aspirate *T. gondii*-PCR negative). In TaqMan-based PCR we

Table 1. Detection of *Toxoplasma gondii* by nested-PCR and Real-time assays

Patient	Specimen	Nested-PCR	TaqMan assay	LightCycler Assay
1	Cerebral biopsy	+	+	+
1	Cerebrospinal fluid*	+	+	+
1	Cerebrospinal fluid*	+^	-^	+^

+: Positive; -: Negative

* Specimens were collected at an interval of one month between each other

^ The assay was re-performed and the result was confirmed

found the same sensitivity, as observed for the blood samples whilst in nested-PCR and in FRET assays we found 10- and 100-fold better sensitivity, respectively (data not shown).

The different sensitivity between FRET- and TaqMan-based Real-time and nested-PCR assays may be explained by the differences in copy numbers of the target sequences (14, 18).

The discrepant result obtained from clinical samples (cerebrospinal fluid samples) by TaqMan-based PCR is probably caused by the copy numbers of the target sequences (13, 17) and, also by the amount of human DNA in the specimens that could have affected the amplification efficiency of the target DNA (18). Moreover, it is noteworthy that TaqMan-based assay, evaluated in this study, was developed and only used by Jauregui (15) for *T. gondii* detection in pig and mouse tissues and not for human samples. At present, this is the first report describing the use of this assay for the detection of *T. gondii* in human samples. In fact, other Authors have developed a single tube nested-PCR to amplify this *T. gondii* 18S rDNA ITS1 region but always for the detection of the parasite in foetal animal tissues (19).

The main advantages of Real-time PCR over nested-PCR assays are that (i) it is far less labor-intensive (only one PCR step, compared to at least two in nested-PCR); (ii) it is performed in a closed system where post-PCR handling is not required (i.e., transfer of amplified template from the primary to the secondary amplification reaction and agarose gel electrophoresis for the detection of PCR products; this constitutes a major advantage since the risks of contamination are minimal); and finally (iii) the result of the assay after DNA extraction can be obtained in only 2 h and 1 h for TaqMan- and FRET-based assay, respectively, versus a minimum of 8 h with nested-PCR.

For a possible application of Real-time PCRs in routine diagnosis, we have not analysed enough clinical samples to formulate a definitive opinion. Certainly, the cost of each assay is also important. We have evaluated the prices of the reagents needed to extract DNA and to perform nested and Real-time PCRs for sample (controls included) excluding the cost for the instruments. We obtained that for the

commercial nested-PCR the cost is about 87.00 € whereas for TaqMan- and FRET-based PCR it is about 26.00 € and 27.00 € respectively. At present, despite the higher cost of reagents, the commercial nested-PCR assay (Amplimedical – Bioline Division) remains the preferable method for laboratories with a low charged-work (about 20 samples for year) because the assay is ready-to use.

We can conclude that Real-time assays, evaluated in this study, are rapid and specific. These methods are easy-to-use, producing results more readily than in conventional PCR systems. However, the target gene chosen can influence the performance of the PCR assays, as we demonstrated.

Real-time PCR protocols should be optimised and carefully evaluated in a larger number of clinical samples before they are implemented as routine methods, but, certainly, these assays for their properties offer a practical and acceptable alternative to nested-PCR for a rapid and accurate diagnosis of toxoplasmosis.

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