

Development of a PCR test for the diagnosis of *Toxoplasma gondii* infections

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SUMMARY

Introduction. Toxoplasmosis is a paucisymptomatic and self-limiting disease in the normoergic patient, but it becomes potentially dangerous in immunocompromised patients (transplanted or HIV-infected patients) and during pregnancy, because if transmitted from mother to fetus can cause serious malformations to the future child and consequences to the newborn. Recent studies evidenced that a proper early administered treatment can reduce the seriousness of symptoms in newborns and immunosuppressed patients. Because the serological diagnosis in these categories of patients is often insufficient, the search of DNA of *Toxoplasma gondii* by Polymerase Chain Reaction (PCR) is progressively used.

Although PCR is a specific and sensitive technique, today a fully standardization is still lacking.

The aim of this study was the development of a new test based on PCR for the detection of the *Toxoplasma gondii* parasite in different biological samples.

Methods. In this study we proved the performances of a new PCR system, with ready-to-use reagents, where the highly repetitive RE region of *T. gondii* (GenBank accession number AF146527) is the target gene. As reference, we used the control panel of the European Molecular Biology QCMD 2008 *Toxoplasma gondii* (TGDNA08) EQA Program. EQA panel for the identification of *T. gondii* consists of eight samples containing different concentrations of the parasite and two negative samples (seven amniotic fluid and three plasma samples).

Results. All negative samples were confirmed, as strong positive too. Only one sample with low concentration (5 parasites/ml) provided positive results in 50% of the ten repetitions of the *intra-assay* test, defining the threshold of sensitivity of the test.

Conclusions. This study, although preliminary, demonstrates the specificity and sensitivity of the new test on different biological samples.

INTRODUCTION

The protozoan parasite *Toxoplasma gondii* is the infective agent responsible for Toxoplasmosis (6, 11). Whilst in the United States it is estimated that 22.5% of the population 12 years and over have been infected, in other places in the world it has been shown that up to 95% of the population have been in contact with *Toxoplasma* (8). The diffusion of the parasite is more represented in hot and humid areas with no high altitudes.

The way of infection is mainly through microscopic cysts included in food that can be transmitted by undercooked, contaminated meat or handling contaminated meat and contact with not washed hands, or by using not washed knives and utensils came in contact with infected meat.

The most important role in the parasitic transmission is played by cats. Cats usually eat rodents, birds, or other small infected animals. The parasites then passed in the cat's feces in a oocyst form (21). The passage from person to person was never evidenced, except in mother-to-child transmission (7, 17), and blood transfusion (5, 9) or organ transplantation (4, 15). The transplantation of an organ from a *Toxoplasma*-positive donor can cause the infection of the recipient, and, in some cases, bring to a severe prognosis.

The most diffused surveillance for *Toxoplasma* infection is in mother-to-child (congenital) transmission (2, 16). A woman who becomes positive to *Toxoplasma* during pregnancy can pass the infection to the fetus.

The mother may not have symptoms, but severe consequences, such as miscarriage or diseases to the nervous system or to the eyes of the unborn child could arise during pregnancy or later in the infant. If the woman had a previous *Toxoplasma* infection before becoming pregnant, the unborn child will be protected, but she can pass the infection if the contact with the parasite is during or immediately before pregnancy.

The earlier the infection is, the more severe is the damage to the unborn child. The diagnosis of *Toxoplasmosis* is typically made by serologic testing (12, 14), but the molecular tech-

niques that are able to detect the *Toxoplasma*'s DNA in the amniotic fluid, or in the peripheral blood of the mother, can be very useful in cases of possible mother-to-child (congenital) transmission (18-20).

OBJECTIVES

The aim of the present study is to introduce a new PCR-based assay for *Toxoplasma* detection able to identify a possible mother-to-child transmission, or an infection derived from a positive organ transplantation, or again from an infected blood transfusion with a sensitivity and a specificity at least equal to the panel of the serological gold standard and direct observation panel tests.

The test involves the extraction of nucleic acids from amniotic fluid, or whole blood, or tissues, and the amplification via a simple PCR protocol. The detection is performed on electrophoretic agarose gel.

The system contains an internal control to assess the functionality of the test, and to exclude PCR inhibition.

DESIGN AND METHODS

The assay is a ready-to-use test that includes all the required elements for the amplification of nucleic acid targets.

Amniotic fluid or whole blood have been extracted with a convenient protocol in order to obtain good-quality DNA.

For this study we used a silica based system developed internally. The extraction protocol starts with a washing step balanced on the different biological material.

Then, a solution containing a chaotropic agent and a suspension of silica for binding the DNA, are added to the washed sample.

The suspension is left 3-5 minutes at room temperature.

A further washing step with ethanol completes the procedure. The pellet is left to dry for five minutes at room temperature, and then dissolved in 100 - 200 µl of sterile water.

The DNA was immediately used for the PCR protocol and then stored at -20°C.

The set of primers for the amplification of *Toxoplasma gondii*

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was designed in the RE region (GenBank accession number AF146527), a repetitive sequence of 200-300 repeats in the *Toxoplasma* genome, giving an amplified fragment of 134 bp. In each test tube an internal control was also included. The primers for the internal control were designed in the human beta-globin gene. The choice for a housekeeping gene was done to introduce both a control of the extraction system and a control for the efficiency of the amplification.

The amplified fragment corresponds to a band of 268 bp. The two sets of primers for the specific region of *Toxoplasma gondii* and for the internal control were designed to have the same thermal profile and to be amplified simultaneously in a multiplex format. Each test was performed using two units of Hot Start DNA Polymerase. The thermal profile of the PCR reaction is described in Table 1. The amplification was performed on an Applied Biosystems Veriti thermal cycler.

The assay was tested on *Toxoplasma gondii* QCMD (Quality Control for Molecular Diagnostics) 2008 panel in collaboration with the Toxoplasmosis laboratory of the Foundation IRCCS Policlinico San Matteo (Pavia – Italy). All the samples of the panel were extracted and tested in blind. The panel of the QCMD 2008 was tested in ten replicates. Three positive malaria samples and three positive Leishmania samples were introduced for the specificity assessment.

The results were evidenced on a 3% agarose electrophoretic gel, after a run of 30 minutes at 120 Volts in TBE 1X buffer. The *Toxoplasma gondii* panel used in this study was obtained from the QCMD 2008 *Toxoplasma gondii* (TGDNA08) EQA Programme. The panel consisted of eight samples containing various concentrations of *T. gondii* and two negative samples (Table 2). The samples were prepared starting from *Toxoplasma* cultures, diluted and resuspended in a matrix. The matrix was clinical amniotic fluid or plasma, previously tested serologically and by molecular techniques (PCR) to ensure negativity for *T. gondii*. The samples from TG08-01 to TG08-07 were dissolved in amniotic fluid. The samples from TG08-08 to TG08-10 were dissolved in plasma.

RESULTS

The panel of samples considered in the present study include strong positive as well as weak positive and negative samples. All the samples were tested in repetitive runs for the intra-assay and inter-assay evaluation.

Qualitative results obtained using primers specific for RE sequence were compared with the expected results of the panel. The results showed a perfect agreement in ten out of ten samples of the panel.

Even in presence of very low levels of parasitic infection (5 parasites/ml in some samples) the developed test was able to detect the presence of *Toxoplasma gondii* in the sample.

In the two lower concentrated samples (5 parasites/ml), the repetitions resulted positive in 50% of cases in amniotic fluid and 60% in plasma samples, indicating this concentration as

a detection limit.

Figure I shows the electrophoretic profile of a PCR run. All the bands corresponding to the internal control (beta-globin) are clearly amplified. The ready-to-use format avoids problems related to cross-contamination due to multi-step preparation of common PCR master mixes.

In order to evaluate the specificity of the test we also used samples positive for other parasitic infections such as Leishmania and Malaria. No false positives were observed in all the checked samples. No cross – reactivity between *Toxoplasma* and other parasites was observed. The specificity of the test was equal to 100% (Figure II).

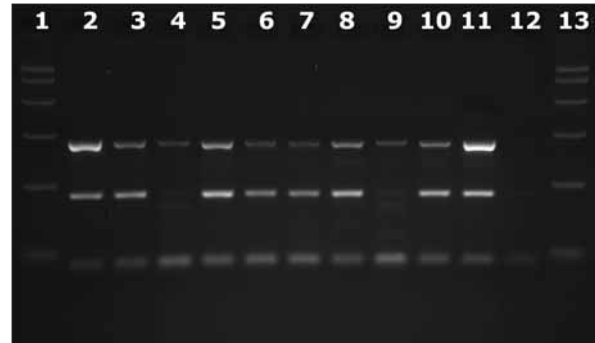


Figure I. Lanes 1 and 13: molecular weight marker (BenchTop Markers – Promega); lanes 2 - 11: *Toxoplasma gondii* (TGDNA08) EQA Panel. Lane 2: TG08-01 sample; lane 3: TG08-02 sample; lane 4: TG08-03 sample; lane 5: TG08-04 sample; lane 6: TG08-05 sample; lane 7: TG08-06 sample; lane 8: TG08-07 sample; lane 9: TG08-08 sample; lane 10: TG08-09 sample; lane 11: TG08-010 sample. Lane 12: negative control (no DNA).

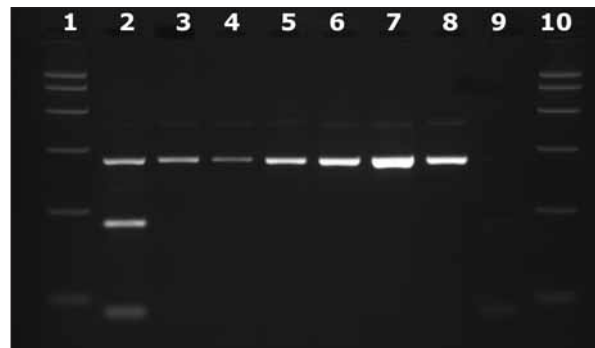


Figure II. Absence of cross-reactivity between *Toxoplasma* and other parasites. Lanes 1 and 10: molecular weight marker (BenchTop Markers - Promega); lane 2: *Toxoplasma gondii* positive sample; lane 3: *Plasmodium falciparum* positive sample; lane 4: *Plasmodium vivax* positive sample; lane 5: *Plasmodium ovale* positive sample; lanes 6 - 8: *Leishmania* spp. positive samples; lane 9: negative control (no DNA).

Table 1. PCR Protocol. The thermal profile and the timing of the amplification protocol are described.

N° of cycles	denaturation	annealing	extension
1	94°C – 2 min		
40	94°C – 30 sec	55°C – 30 sec	72°C – 45 sec
1			72°C – 5 min

Table 2. Panel composition.

Sample	Sample content	Sample matrix	Sample concentration Tg/ml	Sample status
TG08-01	<i>Toxoplasma gondii</i> (RH)	amniotic	5	Positive
TG08-02	<i>Toxoplasma gondii</i> (RH)	amniotic	50	Strong Positive
TG08-03	<i>T. gondii</i> negative	amniotic		Negative
TG08-04	<i>Toxoplasma gondii</i> (RH)	amniotic	100	Strong Positive
TG08-05	<i>Toxoplasma gondii</i> (RH)	amniotic	20	Strong Positive
TG08-06	<i>Toxoplasma gondii</i> (RH)	amniotic	10	Positive
TG08-07	<i>Toxoplasma gondii</i> (RH)	amniotic	50	Strong Positive
TG08-08	<i>T. gondii</i> negative	plasma		Negative
TG08-09	<i>Toxoplasma gondii</i> (RH)	plasma	50	Strong Positive
TG08-10	<i>Toxoplasma gondii</i> (RH)	plasma	5	Positive

CONCLUSIONS

The ready-to-use PCR assay here described is able to detect all the *Toxoplasma* infection of a European reference panel, and to identify all the negative samples without cross-contamination or false positive results.

It could be useful, for increasing the sensitivity of the system to only one parasite/ml, to increase the starting amount of sample for the extraction, or to concentrate the amniotic fluid by centrifugation before extracting.

The test, as used in this study, shows a sensitivity and a specificity of 100% for the *Toxoplasma gondii* parasite.

It can be considered a valuable tool for the diagnosis of subjects suspected to be infected.

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