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A High Sensitive Nested PCR for *Toxoplasma gondii* Detection in Animal and Food Samples

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Abstract

Toxoplasma gondii is a major food and waterborne transmitted parasite world-wide. The tissues and meat samples of many warm blooded animals can contain tissues cysts from chronic toxoplasmosis. Water and vegetable can be contaminated by the parasitic oocysts shed through the feces of infected cats, representing the definitive host of the parasite.

A sensitive PCR for *Toxoplasma gondii* detection is described. The first step amplified the region between the 28S and 18S rDNA in the closely related *T. gondii* and *Neospora caninum*; RFLP analysis distinguished the DNA from the two morphologically identical parasites. Although *N. caninum* is not involved in human transmission, so far, it is important for animal health since is a major responsible for abortion in cattle.

The nested PCR was used in a dilution assay in pork sausage samples spiked with *T. gondii* parasitic DNA. The analysis showed that up to 200fg equivalent to two single parasites only, could be detected. Similar detection limit for *T. gondii* can be obtained with real-time PCRs, but real time methods need special consumables and expensive equipment.

Keywords: *Neospora caninum; Toxoplasma gondii*; Restriction fragments length polymorphism; Nested PCR

Introduction

Toxoplasma gondii is a coccidian protozoan that can infect different tissues in several warm blooded animals and toxoplasmosis is the most diffused food and waterborne parasitic infection worldwide [1]. The serological screening for *T. gondii* reveal high positive level in livestock and the presence of tissue cysts in meat is an important risk factor for toxoplasmosis transmission. The infection is quite often mild or asymptomatic in immune-competent individuals but can be particularly severe in immune-compromised subjects. Furthermore *T. gondii* is implicated in abortion cases in several animal species. Congenital toxoplasmosis (the vertical transmission from the mother to the fetus) is a big concern in public health for the potential severe outcomes for the baby [2]. The consumption of undercooked or raw meat is a main risk factor for toxoplasma infection in human beings, as established by multicenter study [3]. However, several typical cuisines include dishes based on raw meat preparation in many countries and these traditional practices can be difficult to change despite educational campaigns [4].

In this paper, a nested PCR targeting the ribosomal DNA locus is described. The ribosomal locus in *T. gondii* is represented by a 7.5-kb rDNA unit arranged in head-to-tail tandem repeats of at least 110 copies per haploid tachyzoite genome [5]. The sensitivity was evaluated using spiked fresh pork sausage.

Material and Methods

T. gondii DNA was extracted from the reference strain Me49 provided by the European Parasite Reference Laboratory at the National Institute of Health in Rome.

N. caninum DNA was obtained from tachyzoites of Polish bovine isolate NcPolB1 and from a reference strain NC1 maintained at the Witold Stefanski Institute of Parasitology, Warsaw [6].

The analysis on fresh prepared pork sausage was set-up as follows: a sample of 5 g of the sausage was suspended in 5 ml TRIS-EDTA and homogenized by EDTS VIII homogenizer (Design Village Ltd, U.K.). A positive suspension was prepared by adding an aliquot of 10 µl of a DNA solution at concentration of 1 ng/µl to a volume of aliquot of 190 µl of the sausage homogenate. Four serial dilutions of 1 to 10 up to 1 pg of DNA followed by two further dilutions 1 to 5 to a final amount of 0.04 pg were prepared. The DNA was extracted from the homogenates by Gene Elute mammalian kit (cat N° G1N350Sigma-Aldrich St. Louis, MO, USA) following manufacturer's instructions.

First PCR and RFLP analysis

Common primers for *T. gondii* and the related parasite *N. caninum* NC 18S RNA sense primer 5' TGCGGAAGGATCATTCACACG 3' and NC28S RNA antisense primers 5' CCGTTACTAAGGGAATCATAGTT 3' where used for the first PCR. The digestion with endonucleases Hinf I (Promega Madison, WI, USA) of the first amplification product was performed with DNA extracted from the parasite. After first PCR, an aliquot of 5 µl of the PCR reaction was digested in 50 µl of final volume in 1X enzyme buffer at 37°C for 1 hour. The digested products were loaded on 2% agarose gel containing ethidium bromide and visualized on CHEMI-DOC image-analyser (Biorad, Hercules, CA 94547 USA).

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For the nested PCR, 1 µl after the first cycle, was amplified with *T. gondii* specific primers Toxo ITS1sense 5' GATTTGCATTCAAGAAGC(G)TGATAGTAT 3'Toxo ITS1 antisense 5' AGTT(T)AGGAAGCAATCTGAAAGCACATC 3' [7].

Both PCR reactions were performed on 50 µl of 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris-66 HCl (pH 8.4), 100 µM (each) dATP, dTTP, dCTP, and dGTP, 50 pmol of primers, and 3U of Taq gold DNA polymerase (Applied Biosystem, Life Technologies Corporation) with the following PCR program: 10 min at 95°C and then 40 cycles of 50s at 94°C, 30s at 60°C, 1 min. at 72°C and a final extension of 7 min at 72°C in a 9700 thermal cycler (Applied Biosystem Life Technologies Corporation) .

Results and Discussion

The PCR primers used for the first PCR were chosen in a conserved region of the rRNA locus of the two similar parasites *N. caninum* and *T. gondii*. An amplicon of almost 1000 bp is obtained after first amplification spanning the intergenic region included between 18S and 28S of the rRNA locus. The morphologically identical oocysts of *N.caninum* and *T. gondii* can be distinguished by Restriction Fragments Length Polymorphism (RFLP) analyses upon digestion with restriction enzymes. After the restriction with Hinf I, a doublet of 500 bp is present in *T. gondii* and three fragments in *N. caninum* (Figure 1). A comparison of the same region in gene bank database showed that all *T. gondii* and *N. caninum* isolates sequenced so far have the same Hinf I restriction map respectively and different RFLP can be also observed using other frequent cutter enzymes like RsaI and AluI.

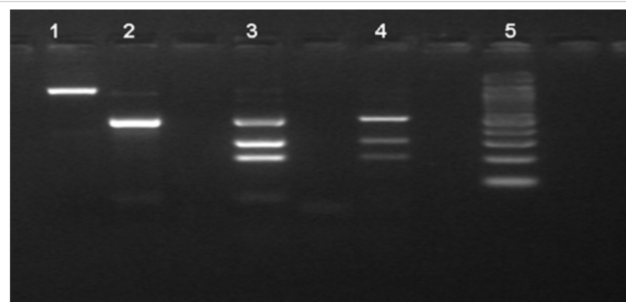

Figure 1: First PCR amplicon of 1000 bp was digested with Hinf I for 1 hour at 37°C and run on 1.5% agarose gel.

RFLP pattern on first PCR-amplicons from *Toxoplasma gondii* and *Neospora caninum* isolates after digestion with Hinf I.

Lane 1: undigested 1 kb amplicon of *T. gondii.*

Lane 2: *T. gondii* me 49, first PCR amplicon digested: the pattern shows a doublet of 500 pb which is indicative of one recognition site for Hinf I.

Lanes 3: digestion pattern in the amplicon of *N. caninum* PolB1 isolate [6].

Lane 4: digestion pattern in the amplicon of NC1 reference strain [6].

Both DNAs show DNA fragments of 500 bp, 300 bp and 200 bp, revealing the presence of two recognition sites

Lane 5: DNA size marker 100 bp ladder.

The two parasites are so closely related and morphological identical that *N. caninum* was misdiagnosed as *T. gondii* until 1988 when its identity and life cycle were discovered [8]. *N. caninum* has a narrower host spectrum and it is not implicated in zoonosis but is important in animal health because it is a major cause of abortion in cattle [9]. Toxoplasmosis on the contrary is the most diffused parasitic zoonosis in the world. Nested PCR, increasing both specificity and sensitivity of the analysis, can be used to screen animal tissues and meat samples such as milk, meat, vegetables, for the presence of the parasitic DNA. The described nested PCR was set on spiked pork sausage because several human acute toxoplasmosis cases in Italy are suspected to be related to this meat preparation. As established by a questionnaire administration, the very common practice to taste raw fresh sausage might be responsible of many cases of acute toxoplasmosis. To evaluate the sensitivity of the method, pork sausage homogenates was spiked with *T. gondii* DNA and a series of dilutions of 1:10 were performed starting from 1000 pg to 1 pg (lane 1 to 4 of figure 2) followed by two dilutions 1:5 up to 40 fg of DNA (lanes 5 and 6). The DNA could be detected in the nested step up to 200fg (lane 5) and no amplification with *N. caninum* DNA was observed (lane 8). The detected amount is equivalent to two parasites with a comparable sensitivity shown in the real-time PCR assay described by Jauregui et al. [7]. Many end-point and real time PCRs have been evaluated for Toxoplasmosis diagnosis so far, but still a unique standardized method is not available [10,11]. Direct comparisons among different methods have shown that the performance of the results depend upon a lot of variables such as the copies number of the DNA template and the type of clinical specimen (blood versus CSF or amniotic liquid) [12,13]. It has also been reported a nested PCR method that had a higher sensitivity compared to two real-time PCRs which allowed a more rapid identification of *T. gondii* DNA but showed some discrepancies in a minority of routine samples from immune-compromised patients [14]. A commercial nested PCR, approved for *T. gondii* analysis in human clinical specimens, has been evaluated for analytical performance and economic impact with two different real-time methods. The analytical performance showed that one real-time method was more sensitive whereas the other real time and nested PCR had the same sensitivity. In terms of economic impact, the commercial nested PCR used by the authors, resulted more expansive in consumables compared to the two real-time PCRs; however nested PCR was still recommended for small laboratories that process few clinical samples per year because of the high cost of the real time equipment [13]. Several nested PCRs for other infectious diseases are often reported to have similar analytical performance to real-

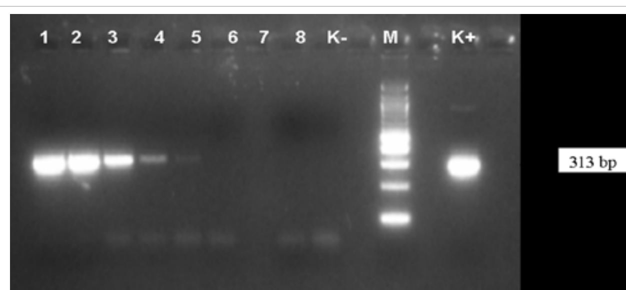

Figure 2: Nested PCR assay with fresh pork sausage samples spiked with *T. gondii* DNA at different concentrations expressed in picograms (pg) and femtograms (fg). *T gondii* specific primers amplify a region of 313 bp in the intergenic region 1 (ITS1) of the parasite ribosomal RNA operon [7] internal to the first amplicon.

Lane 1-1000 pg
Lane 2-100 pg
Lane 3-10 pg
Lane 4-1 pg
Lane 5-200 fg
Lane 6-40 fg
Lane 7-empty
Lane 8-PCR with *Neospora* DNA (NC1)
K-: PCR mix with no template
K+: PCR mix plus *T. gondii* DNA from strain Me49.
The DNA could be detected up to 200 fg corresponding to two parasites (lane 5).
The amplicon of 313 bp is pointed by the arrow.

time PCRs that require higher cost for equipment and consumables [15,16]. The described in house nested PCR in our hands is cheaper in consumables and has the advantages to permit: i) the distinction between *N. caninum* and *T. gondii* oocysts which is important in veterinary medicine and ii) the screening of food samples by a high sensitive method with no need for expensive equipment. Although the nested PCR is more time and work consuming with a higher possibility of sample contamination, it is easily performed in many laboratories in the veterinary field and in developing countries where budget constraints are important.

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