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Polymerase Chain Reaction for Detection of *Toxoplasma gondii* in Human Biological Samples

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ABSTRACT. Using the polymerase chain reaction (PCR), *Toxoplasma gondii* from gene TGR1E with primers TGR1E-1, TGR1E-2 (standard PCR), and from B1 gene with primers TM1, TM2, TM3 (hemi-nested PCR) was detected in biological samples from 347 individuals (441 biological materials). Of the total of 441 biological materials, *T. gondii* DNA was detected in 5.2 %; it was positive in the following samples: blood (n = 6), blood from newborns (2), biopsies (2) and samples of progenitor cells (2) (from candidates for bone marrow transplantation). DNA of *T. gondii* was also revealed in 11 samples (8.3 %) of 120 cases of pregnant women during prenatal examinations. A positive result in the blood was also found in two cases of newborn babies from mothers who were infected in later pregnancy. The positive PCR examination was confirmed by serological methods (ELISA and complement fixation test). Agreement of PCR results and the detection of antibodies against toxoplasma was found in 83.3 %. Rapid PCR examination for the confirmation of acute parasitemia *T. gondii* is particularly important for the patients in whom the infection may cause serious consequences (*e.g.*, for fetus in pregnant women or for patients suffering from imunosuppression).

Infection by the parasitic protozoon *Toxoplasma gondii* is one of the most common parasitic diseases both in the world and in Czechia. Besides human beings, *T. gondii* infects a number of animals (intermediate host of the parasite). The definitive hosts of *T. gondii* are feline predators; the sexual development of parasites is realized in their digestive system. The frequency of the transmission of infection to people depends on the professional and social habits of the population, its composition and life style, such as contact with the feces of infected cats, and the eating of insufficiently processed meat (Čatár *et al*. 1998). Generalized infection may develop in patients who have a serious damage of the immune system of different origin, *e.g.*, caused by immunosuppressive therapy, malignant tumors, hematological disease, HIV infection, *etc*. (Belanger *et al.* 1999).

Infection is especially dangerous in pregnant woman as *T. gondii* can penetrate the placenta and cause damage of the fetus and result in a congenital type of toxoplasmosis (Palička *et al*. 1998; Jenum *et al.* 1998). In Europe, congenital toxoplasmosis afflicts 1–10 fetuses in every 10 000 newborns. Death or developmental problems, particularly in learning, may occur later in 1–2 % of these children. In 4–27 % cases, retinochoroidal lesions may develop, a disorder which can aggravate vision problems.

In laboratory diagnoses, the most frequently used method for the confirmation or elimination of *T. gondii* infection is the detection of serum antibodies by complement fixation test, ELISA tests for detection of the markers of acute infection in IgM, IgA, IgE classes, and antibody detection in IgG class. IgG avidity goes up after an acute phase of infection, ≈4–5 months after contact with antigens of *T. gondii* (Kodym and Tolarová 1998). Methods of direct detection of the causative agent which are based on the infection of tissue culture or sensitive laboratory animal are time-consuming and technically demanding.

At the shared workplace of our two Departments, we introduced the method of polymerase chain reaction (PCR) for *T. gondii* DNA detection from clinical material. The reason was a relatively complicated interpretation of the results of serological examination in different types of toxoplasmosis (cf. Guy and Joynson 1995; Lamoril *et al.* 1996).

MATERIALS AND METHODS

Descriptive characteristics of the group. A total of 441 biological samples (sent in 2000–2004 to our laboratory for *T. gondii* DNA detection from different clinical workplaces in the Czechia) involved 347 individuals (27 men, 318 women and 2 newborn babies). The diagnoses of patients were: 223 differential

diagnoses of toxoplasmosis, 120 toxoplasmoses found at prenatal examinations, 2 newborns with congenital toxoplasmosis, and 2 suspected toxoplasmoses in candidates for bone-marrow transplantation.

Biological material: blood samples (315), amniotic fluid (118), fetal blood (4), biopsy (2), progenitor cells (2) (Burg *et al*. 1989; Guy *et al*. 1996; Pelloux *et al*. 1996).

Polymerase chain reaction for T. gondii *detection* modified in our laboratory. DNA isolation: DNA was isolated from noncoagulated blood, fetal blood, and amniotic fluid by QIAamp DNA Mini Kit (*Qiagen*), and from biopsies by phenol–chloroform extraction. Amplification of two different areas of *T. gondii* genome was used to increase the reaction sensitivity (TGR1E and B1 genes).

TGR1E gene – repetitive 35× in the genome of *T. gondii*; the reaction was performed as multiplex PCR. Primers: TGR1E-1: 5′-ATG GTC CGG CCG GTG TAT GAT ATG CGA T-3′; TGR1E-2: 5′-TCC CTA CGT GGT GCC GCA TTG CCT-3′ (*Generi Biotech*; Lamoril *et al*. 1996).

For the control of polymerase inhibition, co-amplification was used with the part of β-globin gene with primers (*Generi Biotech*) IC329-1: 5′-ACA GAA CTG TGT TCA CTA GC-3′ and IC329-2: 5′-CAT CAG GAG TGG ACA GAT CC-3′.

PCR Master-Mix (total volume 20 μL). Buffer *Gibco* 10× 2.5 μL, 25 mmol/L Mg^{2+} (2.5 μL), 2.5 mmol/L dNTPs 2 μL, primers (10 pmol/μL): 1 μL TGR1E-1, 1 μL TGR1E-2; 1 μL β-globin 1 (10 pmol/μL), 1 μL β-globin 2 (10 pmol/μL), 1 U (0.2 μL) Taq DNA polymerase (5 U/μL; *Gibco*), 8.8 μL aqua p.inj.

PCR conditions (Thermocycler *Perkin-Elmer* 2400): initial denaturation 94 °C 3 min, amplification 40 cycles (94 °C 30 s, 60 °C 30 s, 72 °C 30 s), final extension 72 °C 7 min.

B1 gene – in genome 30–35×, reaction was performed as one-tube hemi-nested PCR (primers TM1, TM2, TM3; Pujol-Riqué *et al.* 1999). *Primers*: TM1: 5′-GAG AGG TCC GCC CCC ACA AG-3′, TM2: 5′-CTG CTG GTG CGA CGG GAG TG-3′ (619-bp product), TM3: 5′-CAG GAG TTG GAT TTT GTA GA-3′ (362-bp product).

PCR Master-Mix (total volume 20 μL). Buffer *TaKaRa* 10× (with 1.5 Mg^{2+}) 2.5 μL, 2.5 mmol/L dNTPs 2 μL, primers: 0.25 μL TM1 (1 pmol/μL), 0.25 μL TM2 (10 pmol/μL), 2.5 μL TM3 (10 pmol/μL), 1 U (0.2 μL) Taq DNA polymerase *TaKaRa*, 12.3 μL aqua p.inj.

PCR conditions (Thermocycler *Perkin-Elmer* 2400): initial denaturation 95 °C 3 min, 30 amplification cycles (94 °C 30 s, 65 °C 30 s, 72 °C 60 s), final extension 72 °C 10 min, and 30 cycles (94 °C 30 s, 55 °C 30 s, 72 °C 60 s), final extension 72 °C 10 min.

Products were detected by electrophoresis (TGR1E in 3 % agarose gel, product size 191 bp; B1 in 2 % agarose gel, product of the size 362 bp), stained with ethidium bromide and visualized under UV light.

Fragment size was determined by comparing with the DNA standard (Marker XIII; *Roche*). Inserted internal controls: positive control – isolated *T. gondii* DNA at a concentration of 10–100 tachyzoites per mL, control of detection limit – isolated DNA at a concentration of 1–10 tachyzoites per mL; negative control – sterile water; inhibition examination – co-amplification of the part of β-globin gene carried out simultaneously with the amplification of TGR1E gene area (multiplex PCR) (Pelloux *et al.* 1996). Limit of reaction detection (Gross *et al*. 1992): 1–5 tachyzoites per mL of liquid biological material.

Serological tests. The detection of anti-*Toxoplasma* antibodies was done in samples of biological material concurrent by the routine complement fixation test (CFT; *KFR Sevapharma*, Czechia) and the ELISA method (*Test-Line Brno*, Czechia) for the detection of separate immunoglobulin classes (IgG, IgM, IgA, IgE and avidity of class IgG).

Statistical evaluation was done by the NCSS 2000 program (nonparametric Mann–Whitney test).

RESULTS

Among the 441 biological samples from 347 individuals DNA of *T. gondii* was detected using the PCR in 23 samples (5.2 %); in 223 samples of differential diagnosis of toxoplasmosis the detection was positive in blood (n = 6), blood of newborns (2), biopsy (2), progenitor cells from patients with immunosuppression before transplantation of bone marrow (2).

T. gondii DNA was detected in 11 samples from 120 pregnant women (8.3 %); the mother's blood was positive in 5 samples, amniotic fluid in 4 samples. Both amniotic fluid and mother's blood simultaneously were positive in 1 case. Positive results were also found in 2 cases of newborns with congenital toxoplasmosis from mothers who were infected in later pregnancy.

T. gondii DNA-positive findings were compared with serological examination in individual patients; consistent results with anti-*Toxoplasma* antibodies in biological material were found in 83.3 %. Positive *T. gondii* DNA in blood of newborns with congenital toxoplasmosis was confirmed by serological findings of high antibody titers in the blood of both newborns (Table I).

Table I. The PCR detection of *T. gondii* DNA (TGR1E, B1 genes) and antibody examination (CFT, ELISA)[a] in 2 newborns with congenital toxoplasmosis

Newborns	TGR1E	B1	CFT	ELISA				
				IgM	IgA	IgE	IgG	IgG avidity
1	+	+	128*	3.010*	4.295*	nd	2.556*	nd
2	+	–	1024*	1.687*	3.744*	1.442*	2.418*	53.8

[a]Limits of positivity for ELISA tests: 1.100 for IgM, IgA, IgE; 1.100 for IgG; exceeding the range of 30.0–35.0 for IgG avidity (after 4–5 months of infection); * – positive, nd – not examined.

We did not find in antibody titers any statistical difference in the group of patients with positive *T. gondii* DNA detection compared with the group of negative findings.

DISCUSSION

It is not possible to define the actual phase of toxoplasmosis on the basis of serological examination exactly and in time (Kodym and Tolarová 1998). At present, when toxoplasmosis is suspected, the first step is usually the taking of blood for serological examination. When the markers of acute infection are found, examination with PCR is carried out, and this confirms any acute process. Laboratory examination is an important part of toxoplasmosis diagnosis but it is necessary, when evaluating the results, to take into consideration even epidemiological implications and the clinical condition of the patient and the course of therapy. Effective antibiotic treatment appears, as in other infections, in quickly breaking down the protozoon *T. gondi*, and eliminating it from the organisms, so the *T. gondii* DNA may not be proved (false-negative result). Therefore, to prevent false-negative results, examination by the PCR method should be indicated before starting antibiotic therapy, immediately after suspicion on *T. gondii* infection is raised. Unfortunately, the PCR results may also be false-negative in certain pregnancies, when the fetus has been damaged (Gratzl *et al*. 1998; Jenum *et al*. 1998).

Correctly indicated examination by PCR for the detection of *T. gondii* DNA is an important part of laboratory diagnosis of toxoplasmosis, particularly for the possible determination of actual parasitemia in pregnant women and patients with immunosuppression (Guy and Joynson 1995; Belanger *et al*. 1999).

Treatment of infected pregnant women with a positive PCR result followed by treatment of their infants is usually successful. In one of our cases, in a 26-year-old pregnant woman a positive finding in the DNA (in blood) and the results of serological examination did not correlate, and the results were negative except for a slightly positive CFT (1 : 8). In this case *T. gondii* infection was diagnosed at the onset before an antibody response developed. Further increase of antibodies (positivity in serological tests), confirmed the hypothesis. This case confirms the significance of including direct DNA *T. gondii* detection in the laboratory diagnosis of toxoplasmosis.

The fast detection of acute parasitemia of *T. gondii* by the PCR method is also significant in pregnancy as toxoplasmosis in gravidity is a serious disease (Graco *et al*. 2003). In the acute stage of toxoplasmosis transplacental penetration of tachyzoites to the fetus environment may occur (Guy *et al*. 1996; Gratzl *et al*. 1998), resulting in infecting the fetus and causing possible permanent damage. Both newborns (*see* Table I) were born to mothers who were examined as late as the third trimester of gravidity. *T. gondii* DNA was shown by the PCR method in the samples of amniotic fluid (Hohlfeld *et al*. 1994). Laboratory examinations of both babies of the infected mothers were done immediately after their birth with a finding of *T. gondii* DNA and positive serological markers of acute infection. High-avidity IgG class immunoglobulin in the examined newborns was probably caused by transplacental passage of maternal antibodies (IgG).

A cranial ultrasound examination of the first child (cf. Table I) was negative; clinical examination showed slight psychomotor retardation. Ultrasound examination of the brain of the second newborn showed a dilatation of the left lateral ventricle and numerous calcifications. Upon examination after the treatment a regression of the dilated lateral ventricle and a significant reduction of calcifications in the brain was observed.

The difficult clinical interpretation of serological findings in patients with a suspected infection, ongoing disease or latent toxoplasmosis was the reason for introducing the *T. gondii* DNA detection by PCR

in our laboratory; by this method, an actual parasitemia in infected organisms could be proved. Serological markers of acute infection in IgM, IgE and IgA classes persist in many patients for a long time (months and years), and it is not possible to determine the actual stage of the disease with certainty. Rapid detection of *T. gondii* DNA (gene TGR1E, B1) by the PCR method is thus very important for fetuses of pregnant women, newborn children with a suspicion of congenital toxoplasmosis (Palička *et al.* 1998) and for patients treated by immunosuppressive therapy (Slavin *et al*. 1994).

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