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CLINICAL STUDY

Molecular diagnosis of *Toxoplasma gondii* in pregnant women

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Abstract: *Background:* Novel approaches in the diagnostics of *T. gondii* have enabled a detection of the parasite in the amniotic fluid or blood of pregnant women.
Objective: The high titres of IgM and IgG antibodies against *T. gondii* are not always indicative of the presence of this parasite in pregnant women, therefore the molecular assays can be used to diagnose and genetically characterise *T. gondii* in amniotic fluids and blood samples.
Methods: The study analysed 15 samples of amniotic fluid and 1 sample of the blood from pregnant women suspected for toxoplasmosis. The serological ELISA test was used for the immunological study and molecular analyses, PCR at SAG2 locus followed by RFLP analysis were used for *Toxoplasma gondii* genotyping.
Conclusion: Using PCR assay with TGR1E gene we have confirmed the presence of *T. gondii* in the blood of a pregnant woman. The parasite was typed as genotype I, belonging to virulent strains *(Tab. 1, Fig. 2, Ref. 30)*. Full Text in PDF *www.elis.sk.*
Key words: *Toxoplasma gondii*, molecular methods, PCR analysis, genotype.

Toxoplasmosis is one of the most common parasitic infections with a worldwide prevalence. Though causing an asymptomatic infection in a healthy host (80 – 90 %), it can be accompanied by non-specific clinical symptoms (swollen lymph nodes in the neck area, fewer, head and muscle aches, increased fatigue). Rarely, an immunocompetent individual may develop distinct symptoms, e.g. generalised lymphadenopathy, maculo-papular exanthema, hepatitis, encephalitis, myocarditis, however, in very rare cases, the infection can be fatal (1, 2). The *incubation period* for toxoplasmosis is usually 4 – 21 days (with an average of 7 days) (3). Clinical manifestation of *T. gondii* infection depends on the host-parasite interaction, virulence, infective dose, host immunocompetence and age (4).

Toxoplasmosis present a serious health threat to immunocompromised patients and pregnant women that were infected for the first time immediately prior to or during pregnancy.

Toxoplasmosis is a common opportunistic infection in immunosupressed individuals, in particular AIDS patients (5, 6, 7), organ transplant recipients (8) and in patients with malignant diseases (9). Bradyzoites-to-tachyzoites conversion in AIDS patients results in encephalitis (10) with the prevalence of 25 % (11) and also in pneumonia and chorioretinitis. *T. gondii* infection can be activated by cysts after an organ transplant, or by tachyzoites transmitted through blood transfusion. Immunosuppressed patients are at a risk for reactivation of latent toxoplasmosis (12). Negative pregnant women are especially at risk for a vertical transmission to foetus. In such cases, *T. gondii* infection could induce a life-threatening disease in foetus (13).

Encysted bradyzoites that persist in the tissue cysts, localized in the CNS, heart and skeletal muscles are distinctive for latent toxoplasmosis (14). They induce immune response that persists throughout the lifetime of the host (8). Bradyzoites in the tissue cysts are a source of infection when transmitted to humans from domestic animals. In immunodeficient individuals, the release of bradyzoites and subsequent transformation to tachyzoites may result in fatal *Toxoplasma* encephalitis (15, 9, 16).

The aim of the study was to detect *T. gondii* in the blood and amniotic fluid samples of pregnant women and genetically characterise the causative agent of toxoplasmosis.

Material and methods

In co-operation with several medical facilities (Louis Pasteur University Hospital, Kosice) a total of 16 samples of amniotic fluid and blood of pregnant women were collected. ELISA tests confirmed high titres of IgG and IgM antibodies specific to *T. gondii*. Isolated DNA of *T. gondii* was stored at –20 °C until further use.

ELISA test was used for a serological detection of the specific antibodies in sera (IgG, IgM) in the stage of an acute infection. The serological diagnosis is not that reliable in the case of toxo-

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Tab. 1. Pregnant women examined for the presence of *Toxoplasma gondii*.

No.	ELISA		Material	Molecular analysis PCR (TGR1E gene)
	IP – IgM	IP – IgG		
1.	1.20	1.82	AT	neg.
2.	1.60	3.25	AT	neg.
3.	1.60	3.24	AT	neg.
4.	1.41	1.72	AT	neg.
5.	1.52	2.16	AT	neg.
6.	1.25	1.75	AT	neg.
7.	1.83	2.01	AT	neg.
8.	1.91	2.12	AT	neg.
9.	1.58	1.93	AT	neg.
10.	1.24	1.48	AT	neg.
11.	1.33	1.76	AT	neg.
12.	1.89	2.13	AT	neg.
13.	1.51	1.99	AT	neg.
14.	1.19	1.53	AT	neg.
15.	1.16	1.49	AT	neg.
16.	3.20	5.60	blood	pos.

AT – Amniotic fluid; IP – Positivity Index: IgM > 1.1 = positive IgG > 1.1 = positive

plasmosis, the presence of *T. gondii* can be directly confirmed only by using molecular methods, based on the DNA of parasite.

Amniotic fluid (5 ml) was centrifuged at 95 – 210 x *g* (Jouan BR4i, France) for 10 minutes at a standard laboratory temperature. For *T. gondii* isolation, sediment was diluted with 200 µl PBS (phosphate buffer) and used for the DNA isolation (2).

Blood sample of a pregnant woman was collected into an EDTA tube and incubated in a water bath for 30 minutes at 37 °C. The phase, containing plasma, proteins and leucocytes was removed to 1.5 ml tube and centrifuged at 94 x *g* for 10 minutes. For DNA isolation, sediment containing leucocytes and *T. gondi* was used.

Commercial Kit QIAamp® DNA Mini Kit (QIAGEN, GmbH.,Hilden, Germany) was used to isolate *T. gondii* DNA from amniotic fluid and blood. The DNA was isolated according to the manufacturer's protocol, using proteinase K digestion. The final DNA volume was diluted in150 µl of water (Water, nuclease–free, Fermentas) and stored at –20 °C until further use.

The isolated DNA were analysed by PCR method with two primers of TGR1E gene according to Lamoril (17) and Čermáková (2). Amplification was performed in 5 µl of the isolated DNA template. The positive fragment after amplification consisted from 191 bp electrophoresed on a 3 % agarose gel stained with GoldView™ (SBS Genetech, China) and visualized under UV light at 254 nm.

Genotyping of DNA samples of *T. gondii* were performed using the nested PCR at *SAG2* locus according to Howe (18), located at chromosome VIII and the coding surface antigen P22. Samples were analyzed separately at the 5' and 3' regions of the locus, two set of primers were used in two amplifications.

For the RFLP analysis, the fragments after second amplification at the *SAG2* locus, 5´end 241 bp fragment and 3´-end 221 bp fragment were used. The amplified fragments were digested with the restriction endonuclesaes *Sau*3A I (Promega, USA) at 5´ end and *Hha* I (Promega, USA) at 3´ end of locus. The samples were incubated for 3 hours at 37 °C according to Howe at al (1997) and reaction was finished by cooling to 10 °C. The position of the

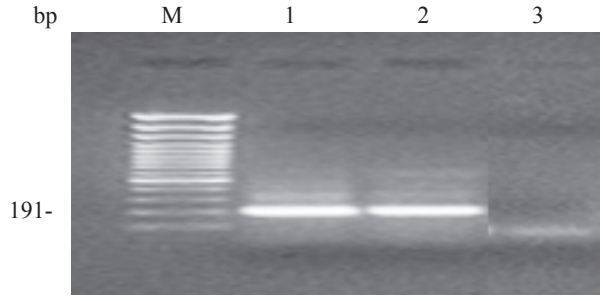

Fig. 1. Diagnosis of *T. gondii* in blood of pregnant women using TGR1E gene. M – marker (Promega); 1 – positive DNA control; 2 – *T. gondii* DNA isolated from blood of positive patient; 3 – negative control.

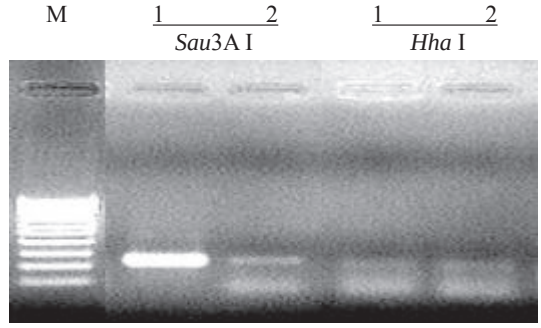

Fig. 2. *T. gondii* genotyping using RFLP at *SAG2* locus from blood of pregnant women. M – marker (Promega); Sau3A I (fragment positive at 241 bp): 1 – positive DNA control; 2 – *T. gondii* DNA isolated from blood of positive patient; *Hha* I (fragment positive at 221 bp): 1 – positive DNA control 2 – *T. gondii* DNA isolated from blood of positive patient.

electrophoresed fragments at 5' and 3' ends visualized on 1.2 % gel determined the genotype of *T. gondii*.

Results

As there is a high risk for *T. gondii* tachyzoites transmission from an infected mother to foetus, causing foetal damage, our research has been focused on detecting *T. gondii* in the amniotic fluid and blood of pregnant women.

A total of 15 amniotic fluid samples, collected at several medical facilities and a single blood sample, collected into EDTA, were used for the *T. gondii* analyses. The ELISA tests were positive in all blood sera of pregnant women (IgG and IgM). Molecular diagnosis of *T. gondii* in amniotic fluid samples was performed by the PCR method using TGR1E sequence. The presence of *T. gondii* was not confirmed in any of the samples (Tab. 1).

T. gondii was detected only in the unclotted blood sample of a pregnant woman with high antibody titres. PCR assay using TGR1E sequence was positive and (Fig.1) and typing at the *SAG2* locus revealed the presence of genotype I (Figs 1 and 2), which is a virulent *T. gondii* strain.

Discussion

As pregnant women are the most vulnerable group when *T. gondii* infection is concerned, they have received the greatest at-

tention in the study of toxoplasmosis. Primary infection during pregnancy may result in congenital toxoplasmosis, accompanied by severe damage to foetus or even miscarriage (16).

Toxoplasmosis, diagnosed in pregnant women, should be always treated, however, an early diagnosis is a key factor for the treatment efficacy. More than 90 % of acute *T. gondii* infections are asymptomatic (19), therefore determining primary infection solely on the basis of clinical signs during pregnancy is rather challenging. Ondriska (12) suggested that serological testing could be performed at the beginning of pregnancy, when the foetus is the most vulnerable and particularly prone to damage. For pregnant women with negative antibody titres, as frequent as in each term of the pregnancy, serology testing is advisable (20), as the infection may be acquired even during pregnancy. Studeničová (21) support the regular testing, as pregnant women lack the protection and are exposed to the *T. gondii* primary infection and potential congenital transmission to foetus.

T. gondii prevalence in human population has been associated with an exposure to risk factors, i.e. hand-to-mouth contact with contaminated soil, ingestion of raw or undercooked meat, contaminated vegetables, poor personal hygiene, unhygienic food processing (22).

During 2003–2007 in Slovakia, 1 201 new cases of toxoplasmosis have been diagnosed, comprising 40.80 % of males and 59.20 % of females. The Regional Public Health Authorities, which have distinguished 9 forms of toxoplasmosis, reported glandular form in 80.0 % of the cases, ocular form in 5.75 %, gynaecological form in 5.66 %, asymptomatic in 3.58 %, febrile form in 1.91 %, lung form in 13 cases, neonatal in 11 cases and dermatological and intestinal form in 7 cases. As much as 72.11 % of the patients recorded in their anamnesis a contact with animals, 3.91 % of the patients ingestion of raw meat, 2.16 % drinking raw milk and in 21.82 % of the patients the anamnesis were unknown. The infection occurred throughout the year, with the highest frequency in January and the lowest in December. The highest age-specific morbidity was found in the age group of 10–14 year old children (23). It may be due to the fact such this age group come into regular and close contact with cats and dogs (24).

In 2008, 175 new cases have been reported (morbidity of 3.24/ /100 000), out of which 62 were males (morbidity of 2.36) and 113 females (morbidity of 4.07). The infections have been reported from all regions in Slovakia, with the highest morbidity in the Trenčín region – 9.84 (59 cases) and the lowest in the Bratislava region – 0.82 (5 cases). The infections occurred throughout the whole year with the maximum in January (42 cases), were reported in all age groups and the highest age-specific morbidity was found in teenagers aged from 15 to 19 years (6.39). Following clinical forms of the infection have been detected: glandular form – 123 cases, asymptomatic – 31, ocular – 9, unknown – 5, gynaecological – 4, intestinal – 1, cardiac – 1 and dermatological 1 case. Epidemiological anamneses in 119 cases stated a contact with domestic animals (contact with various domestic animals – 44 cases, with a cat – 35, with a dog and a cat – 34, with a dog– 6 cases), in 9 cases a contact with a cat and ingestion of undercooked meat and ingestion of raw goat milk, in one case ingestion of raw goat milk, in 8 cases contact with domestic animals, hand-to-mouth contact with soil in a playground, poor hygiene. In 35 cases, the epidemiological anamnesis was unknown (25).

An early and correct diagnosis is essential for the efficient treatment. Positive or negative PCR result favours the decision –making regarding the specific therapy (26). So far, no efficient medication is available to eliminate tissue cysts as bradyzoites in cysts are protected against such medications by the tissue cysts membrane. Current therapy has therefore been focused on tachyzoites. The selected groups of patients with positive results receive sulfadiazine and pyrimethamine. If the results are negative, the spiramycine is recommended as a prevention (2). A suitable human vaccine against *T. gondii* has not been developed yet (27).

Using the molecular methods, *T. gondii* has been detected only in one blood sample of a seropositive pregnant woman. Serological examination of this sample confirmed the presence of IgM and IgG antibodies. The patient's personal anamnesis revealed the fact that her pregnancy had been achieved through assisted reproduction, she had had close and frequent contact with domestic animals (cats, dogs, poultry, goats) and that she had suffered from headaches. *T. gondii* isolated from the blood samples was genetically characterised. This is the first detection of *T. gondii* isolated from blood using molecular methods in Slovakia. The diagnosis of an acute toxoplasmosis was confirmed, caused by genotype I.

Ho–Yen (28) suggested that the samples should be collected prior to antibiotic treatment, but not later that 2 to 3 days after the first examination. Antibiotic treatment usually leads to a rapid elimination of the parasite and the DNA degradation, thus producing false negative PCR results (2). These data correlate with our findings resulting from examining all samples of amniotic fluid, where the patients received antibiotic treatment immediately after detecting high *T. gondii* antibody titres.

Amniotic fluid studies, preferably PCR amplification of *T. gondii* DNA, are the best diagnostic tools for the detection of congenital toxoplasmosis during pregnancy (29). PCR diagnosis from the amniotic fluid largely depends on the gestational age, at which the mother acquired the infection. According to Romand (30), the sensitivity of such prenatal diagnosis is significantly higher if the mother acquired the infection between 17 and 21 weeks of gestation compared to infection acquired before the week 17 and after the week 21 of gestation ($p<0.02$). Romand (30) found, examining 270 amniotic fluid samples, the PCR sensitivity of 64 % and specificity of 100 %.

Conclusion

The serological diagnosis of toxoplasmosis has been known and utilized for a long time, however, implementing new molecular approaches (PCR assays), genotyping and determination of the causative agent virulence, facilitate an early and targeted treatment, thus preventing a damage to a foetus.

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