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Molecular Detection and Genotyping of *Toxoplasma gondii* from Clinical Samples

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<http://dx.doi.org/10.5772/50830>

1. Introduction

Over the past two decades, molecular diagnosis of toxoplasmosis, which is based on the detection of *T. gondii* DNA in clinical samples, became an indispensable laboratory test. This method is independent of the immune response, and depending on methodological approach, may facilitate more accurate diagnosis, especially in cases in which inadequacy of conventional methods is faced with deteriorating and potentially severe clinical outcome (congenital, ocular toxoplasmosis and cases of immunosuppression).

Molecular methods based on polymerase chain reaction (PCR) are simple, sensitive, reproducible and can be applied to all clinical samples (Bell and Ranford-Cartwright, 2002; Contini et al., 2005; Calderaro et al., 2006; Bastien et al., 2007). These methods are divided into two groups. The first group consists of techniques focused on detection of *T. gondii* DNA in biological and clinical samples, including conventional PCR, nested PCR and real-time PCR. The second group consists of molecular methods including PCR-RFLP, microsatellite analysis and multilocus sequence typing of a single copy *T. gondii* DNA and those are predominantly used for strain typing (Su et al., 2010).

However, it is important to emphasize that molecular diagnostics, being a constantly improving modern methodology, is not standardized even among the world's leading laboratories. The differences are substantial and numerous, and they extend to all segments of the methodology such as target genes for parasite detection and markers for genotyping, equipment manufacturers and different protocols (various sets of primers and probes and their concentration, different internal controls, etc...).

2. Molecular diagnostics

2.1. Methodology

Conventional PCR was, in the beginning, the molecular detection method of choice for the majority of laboratories dealing with the diagnosis of toxoplasmosis and it was based on both in-house protocols and commercial kits (Lavrard et al., 1995). To increase the sensitivity of molecular diagnostics of toxoplasmosis nested PCR was introduced, although in recent years real-time PCR has shown a significantly higher sensitivity as well as specificity (Jauregui et al., 2001; Reischl et al., 2003; Contini et al., 2005; Calderaro et al., 2006; Edvinsson et al., 2006). Real-time PCR detection also has the capability of quantification of *T. gondii* in biological samples, which has found wide application in monitoring the kinetics and outcome of infection in patients undergoing therapy, as well as in experimental models (Lin et al., 2000; Jauregui et al., 2001; Contini et al., 2005; Djurković-Djaković et al., 2012).

Molecular diagnostics of toxoplasmosis is generally based on the detection of a specific DNA sequence, using different assays and protocols, mostly from highly conserved regions such as the B1 gene repeated 35 times in the genome, 529 bp repetitive element with about 200-300 copies in the genome, ITS-1 (internal transcribed spacer) that exists in 110 copies and 18S rDNA gene sequences (Table 1). Qualitative PCR protocols for the detection of single copy genes such as the P30 gene appeared less sensitive and they are rarely used for diagnostic purposes (Jones et al., 2000).

Markers	No. of copies	References
B1	≈ 35	Wahab et al., 2010 Correia et al., 2010 Okay et al., 2009
529 bp (AF146527)	200-300	da Silva RC et al., 2011 Yera et al., 2009 Vujančić et al., 2011
ITS-1 or 18S rDNA	≈110	Truppel et al., 2010 Miller et al., 2004
P30	Single copy gene	Buchbinder et al., 2003 Eida et al., 2009 Cardona et al., 2009

Table 1. *T. gondii* DNA detection markers (latest and most significant data)

The first protocol for molecular detection of *T. gondii*, for conventional PCR targeting B1 gene, was developed in 1989 and has since been modified and optimized in many laboratories (Burg et al., 1989; Lopez et al., 1994; Liesenfeld et al., 1994; Reischl et al., 2003; Switaj et al., 2005). The B1 gene, although of unknown function, is widely exploited in a number of diagnostic and epidemiological studies because of its specificity and sensitivity. There are also some studies in which the detection of *T. gondii* parasites was based on amplification of ITS-1 and 18S rDNA fragments, whose sensitivity was similar to the B1

gene (Hurtado et al., 2001; Calderaro et al., 2006). However, the repetitive element of 529 bp in length, which was firstly identified by Homan, has showed a 10 to 100 times higher sensitivity compared to the B1 gene (Homan et al., 2000; Reischl et al., 2003). Nevertheless, there are several studies indicating that there are *T. gondii* strains in which either the whole or parts of the 529bp fragment have been deleted or mutated or in which the number of repeats vary. One report suggests that the 529bp repeat element, of unknown function as well, was not present in all isolates analyzed; 4.8% of the samples gave false-negative results compared to results from amplification of the B1 gene (Wahab et al. 2010.). Furthermore, some of the latest studies question its validity for quantification in clinical diagnostics since the number of copies of the 529 bp repetitive sequence in *Toxoplasma* genome appears to be 5 to 12 times lower than the previous estimations (Costa & Bretagne, 2012). Nevertheless, the detection of *T. gondii* DNA using the 529 bp repetitive element, and real-time PCR protocols that detect the presence of this element, is currently the most widely used molecular approach for the detection of *T. gondii* (Reischl et al., 2003; Kasper et al., 2009).

However, it can be of great methodological significance to further clarify the specificity of using a multicopy target of unknown function before the introduction of such protocol into the laboratory diagnostics (Edvinsson et al., 2006)

2.2. Clinical significance in various biological samples

Molecular detection of *T. gondii* in cases of suspected congenital toxoplasmosis may be performed in the amniotic fluid, and fetal and neonatal blood samples. Also, it is performed in the peripheral blood of immunosuppressed patients, and in samples of humor aqueous and cerebrospinal fluid of patients suspected of ocular and cerebral toxoplasmosis, as well as in bronchoalveolar lavage fluid (BAL). Furthermore, in our laboratory, peripheral blood of patients suspected of acute toxoplasmosis was also analyzed (Table 2).

Clinical sample	Real-time PCR	
	No. tested	No. positive (%)
Blood	91	28 (30.8)
Amniotic fluid	28	10 (35.7)
Fetal blood	9	3 (33.3)
BAL	1	1 (100)
Aqueous humor	10	6 (60)
Cerebrospinal fluid	7	4 (57.1)
Total	146	52 (35.6)

Table 2. Real-time PCR results on various clinical samples of patients suspected of active toxoplasmosis examined in the National Reference Laboratory for Toxoplasmosis, Belgrade, Serbia

2.2.1. Peripheral blood

In our laboratory, the presence of *T. gondii* DNA was shown in about one-third (31%) of all analysed cases. Similar research carried out on peripheral blood samples derived from patients with an acute *T. gondii*-related lymphadenopathy, resulted in the detection of parasite DNA in 35% of all samples (Guy & Joynson, 1995). In a study involving patients with acute toxoplasmosis in southeastern Brazil, the rate of parasite DNA-positive peripheral blood samples was 48.6% (17/35) (Kompalic-Cristo et al., 2007). Interestingly, both of these studies used B1 as the target gene, which is considered to be of lower sensitivity than the AF146527 marker that we used; however, the total number of analyzed samples was smaller and the patient selection criteria may have differed. In addition, in the Brazilian study DNA extraction was performed from the buffy coat instead of the whole blood, which may have contributed to the extraction of larger amounts of parasite DNA and higher success in its detection (Menotti et al., 2003; Jalal et al., 2004). Timely (early) sampling is also of particular importance, as detection of *T. gondii* DNA from the peripheral blood of patients with acute toxoplasmic lymphadenopathy has been shown to be very difficult 5.5 to 13 weeks after the onset of infection (Guy & Joynson, 1995). Successful PCR detection of *T. gondii* DNA should indicate recent infection. However, one must take into consideration that PCR detection of parasitic DNA alone does not necessarily mean that parasites are viable. The immune system rapidly kills circulating parasites but the *T. gondii* DNA could be retained for some period of time in the circulation. Also, it has been suggested that even in the chronic phase of infection it is possible, though very rarely, to detect in blood the DNA originating from cysts present in the muscles and nervous system (Guy & Joynson, 1995). On the other hand, negative PCR cannot exclude recent infection because of several reasons such as the small number of parasites circulating in the blood or short period of duration of parasitemia. Here it must be stressed that the exact kinetics of parasites in humans still remains unclear. Other reasons for negative PCR results include the small sample size from which DNA is extracted compared to the total volume of blood in the human body, as well as the fact that the blood contains components that may inhibit the PCR reaction, primarily heme, hemoglobin, lactoferrin and immunoglobulin G.

We have analyzed real-time PCR results from peripheral blood samples originating from patients suspected of acute toxoplasmosis according to the serological criteria for acute infection, i.e. avidity of specific IgG antibodies and the finding of specific IgM antibodies. The results showed that positive real-time PCR correlates better with the finding of specific IgM antibodies, than with low avidity of specific IgG antibodies (Vujanić, 2012).

Comparison of molecular detection and bioassay findings on peripheral blood samples of the patients with specific IgM antibodies and specific IgG antibodies of low avidity, suggesting acute toxoplasmosis, has been done as well. It was shown that in nearly one-third (29%) of the analyzed cases *T. gondii* DNA was detected in comparison to approximately 20% positive bioassays. This result undoubtedly indicates a higher sensitivity of the real-time PCR method in relation to the bioassay. However, molecular detection of parasite DNA in peripheral blood is of the greatest significance in immunosuppressed patients, where it may be the only method for both the diagnosis and monitoring of the

therapeutic effect of the administered antiparasitic drugs. The significance of real-time PCR for monitoring the kinetics of the infection has been shown in immunosuppressed patients after bone marrow and liver transplantation (Costa et al., 2000; Botterel et al., 2002 ; Edvinsson et al., 2008; Daval et al., 2010). Using real-time PCR, we too have observed a decline in parasitemia in a patient with reactivated toxoplasmosis during specific treatment (Vujanić 2012).

Although the detection of parasite DNA in peripheral blood of adults may not always be direct evidence of active parasitemia, *T. gondii* DNA detected in fetal and neonatal blood samples is of the utmost clinical importance because there is no possibility of detection of DNA from earlier infections. Therefore, the most important application of molecular methods is in the diagnosis of congenital toxoplasmosis, as the isolation of the parasite in cell culture is insufficiently sensitive (Thulliez et al., 1992; Foulon et al., 1999) and the isolation by bioassay takes approximately 6 weeks.

2.2.2. Amniotic fluid

In the last two decades, the detection of *T. gondii* DNA in the amniotic fluid has become particularly important, as it allows for timely diagnosis of fetal infection, and subsequent implementation of appropriate therapy and infection control (Menotti et al., 2010; Wallon et al., 2010). We have so far studied a total of 28 amniotic fluid samples obtained from women suspected of infection in pregnancy. Real-time PCR revealed parasite DNA in 36% of the amniotic fluid samples whereas mouse bioassay was positive in 25%. A similar difference in the positivity rate between PCR (17/85, 20%) and bioassay (14/85, 16.5%) results was obtained in a study in Egypt (Eida et al., 2009).

Given that in many published studies real-time PCR and bioassay results from the amniotic fluid did not match, which is the case in our research as well, and as congenital infection cannot be excluded by negative PCR (Romand et al., 2001; Golab et al., 2002), for prediction of congenital toxoplasmosis it is optimal to combine both molecular detection and bioassay. In one study of prenatal diagnosis of congenital toxoplasmosis in patients from 6 European centers of reference it was shown that PCR from amniotic fluid has a higher sensitivity (81%) in regard to both bioassay (58%) and cell culture (15%) (Foulon et al., 1999). The combination of PCR and bioassay increases the sensitivity to 91%, and represents the best diagnostic approach (Foulon et al., 1999).

In European countries such as France and Austria regular serological monitoring of pregnant women for *T. gondii* is regulated by law, which allows for precise timing of seroconversion and timely prenatal diagnosis of fetal infection. This has also allowed for a vast experience with the diagnosis of congenital toxoplasmosis, and provided data on the superior sensitivity of molecular methods compared to conventional parasitological tests. Thus, one long-term study conducted in France showed that out of 2632 women in whom the infection occurred during pregnancy, congenital toxoplasmosis was confirmed by positive PCR in the amniotic fluid and/or fetal blood in 34 cases in which congenital infection was diagnosed by conventional methods, as well as in three fetuses in whom the

infection was not diagnosed by other methods (Hohlfeld et al., 1994). Also, in a similar study in Austria, outcome of prenatally diagnosed children was followed-up during the first year of life to assess the validity of PCR results from the amniotic fluid. Of the 49 amniotic fluid samples analyzed, congenital infection was confirmed postnatally by serological monitoring in all 11 (22.4%) PCR-positive ones, whereas none of the 38 children in whom PCR of the amniotic fluid was negative was shown to be infected (Gratzl et al., 1998).

2.2.3. Cord blood

Cord blood is not considered the ideal sample for prenatal diagnosis of congenital toxoplasmosis. For example, the results of a survey carried out in France did not show any positive PCR result among 19 tested cord blood samples from children with proven congenital toxoplasmosis (Filisetti et al., 2003). Nevertheless, cord blood samples that are occasionally provided to our laboratory, have shown a rate of positivity in real-time PCR of 33%.—All cord blood samples in our study were inoculated into mice and the rate of positivity of bioassay was 55.5%. A higher rate of isolation of viable parasites by bioassay compared to the detection of parasitic DNA by real-time PCR may be explained by a larger sample volume used for mouse inoculation in comparison to the amount used for DNA extraction, as well as by probable presence of PCR inhibitors. In one study performed on a representative sample of pregnant women in China a similar rate of real-time PCR positive results was obtained from the amniotic fluid and fetal blood samples (Ma et al., 2003).

It can be concluded that the diagnosis of congenital toxoplasmosis from fetal blood samples should be based on the results of both bioassay and molecular detection.

2.2.4. Aqueous humor

Prior to the introduction of molecular methods, the laboratory diagnosis of ocular toxoplasmosis has been based primarily on a comparison of the level of antibodies detected in the humor aqueous and serum in order to detect intraocular synthesis of specific antibodies (Witmer-Goldman's coefficient). Lately, molecular methods are becoming a standard diagnostic approach in the diagnosis of ocular toxoplasmosis as well. A number of studies has already shown that a positive PCR result is not always accompanied by positive serology indicating local synthesis of IgG antibodies (Villard et al., 2003; Talabani et al., 2009) and thus can be the only confirmation of the diagnosis (Okhravi et al., 2005).

We have so far studied 10 humor aqueous samples from patients clinically suspected of ocular toxoplasmosis of which 60% (6/10) were real-Time PCR positive. A similar result was obtained in a French study when 55% (22/40) of humor aqueous samples were positive by real-Time PCR using AF146527 as a marker (Talabani et al., 2009). Also, the detection of the same AF146527 marker by real-Time PCR in another French study, revealed somewhat lower rate of positive samples, 38.2% (13/34) (Fekkar et al., 2008). It is interesting that in the latter study the sample volume of 10 μ L used for DNA extraction was unusually small, which certainly could affect the success of PCR reactions. However, in another study

performed in Strasbourg, the amplification of 18S rRNA and B1 gene by conventional PCR resulted in the 28% (5/18) of the humor aqueous samples positive for the presence of *T. gondii* DNA (Villard et al., 2003).

2.2.5. Cerebrospinal fluid

Cerebral toxoplasmosis usually affects immunosuppressed patients and is mostly the result of reactivation of chronic infection which may be fatal if left untreated. Definitive diagnosis of toxoplasmosis can be made by the detection of tachyzoites in brain tissue samples obtained by biopsy, but this method, because of its invasiveness, is seldom applied, and certainly not since the PCR, giving consistent and quick result, has been introduced in the diagnostics (Vidal et al., 2004). A study of cerebral toxoplasmosis in HIV-infected patients infected in Brazil, showed that 27.4% (14/51) of cerebrospinal fluid samples were positive for *T. gondii* DNA (Mesquita et al., 2010). Noteworthy, DNA extraction was performed using phenol-chloroform method, in which the phenolic residues can often inhibit the PCR reaction. In our limited experience, of the 7 cerebrospinal fluid samples obtained from patients with different neurological conditions (including one case of congenital hydrocephalus) examined by real-time PCR, 4 (57%) were positive.

2.3. Comment

In summary, all above-mentioned results confirm the value of the use of molecular methods, due to their high sensitivity and specificity, in the diagnosis of toxoplasmosis. Coupled with conventional parasitological diagnostic methods, PCR-based methods allow for the timely diagnosis especially of congenital toxoplasmosis and of reactivated toxoplasmosis in immunosuppressed patients. Further advances of the technology itself along with its wide, (universal) use may be expected to markedly improve diagnostics and monitoring of the course of infection as well as of the therapeutic effect.

3. Genotyping

In the early days of strain designation, isolates of *T. gondii* have been grouped according to virulence in outbred mice. First phylogenetic studies of *T. gondii* strains indicated that their genetic complexity was much smaller than expected (Darde et al., 1992; Sibley & Boothroyd, 1992). Howe and Sibley's *T. gondii* population structure study (1995) performed on 106 isolates collected from both humans and animals from North America and Europe, showed the presence of three clonal types (type I, II and III) and very small differences between clonal lineages which is why it was concluded that *T. gondii* has a clonal population structure. Comparative sequence analysis of individual genes indicated extremely low allelic diversity within the clonal lines, and only 1% divergence at the DNA level. In addition, limited genetic diversity between and within clonal lines indicated that they have quite recently evolved from a common ancestor, 10,000 years ago at the most (Su et al., 2003).

Nevertheless, most recent phylogenetic studies indicate that the population structure of *T. gondii* is much more complex than initially considered. While it has been undeniably established that type II is predominant in Europe and North America (Darde et al., 1992; Howe & Sibley, 1995; Howe et al., 1997), there are significant regional differences. Thus, research in Portugal and Spain showed the presence of types I and III in this area (Fuentes et al., 2001; de Sousa et al., 2006), while genotyping of isolates from Crete and Cyprus showed the predominance of type III (Messaritakis et al., 2008); however it must be noted that these studies have been conducted using only one marker (SAG2 or GRA6). Also, phylogenetic analyses of *T. gondii* isolates, which have only recently begun in South America, Asia and Africa, have shown considerable genetic diversity of this parasite strains.

A realistic picture of the distribution of genotypes in Europe is also difficult to obtain because research on *T. gondii* is not performed to the same extent and using the same methods in all geographical areas. So far, the largest number of isolates has been genotyped in France, mainly thanks to the mandatory program of testing of pregnant women for toxoplasmosis in this country, which allows for the availability of research material. One French study has shown that of the 86 isolates from cases of suspected and confirmed congenital toxoplasmosis 85% were of type II (Ajzenberg et al., 2002). A predominance of the same type was indicated in Poland, where genotyping was also performed in samples originating from clinical cases of congenital toxoplasmosis (Nowakowska et al., 2006). In South-East Europe the first strain genotyped was isolated from a case of congenital toxoplasmosis in Serbia, and was also designated as type II (Djurković-Djaković et al., 2006).

Further work on the genotyping of *T. gondii* strains in Serbia showed another two type II isolates, originating from a case of congenital toxoplasmosis and a case of toxoplasmosis in pregnancy, respectively. However, another isolate from a peripheral blood sample of a neonate with suspected congenital toxoplasmosis had been typed to the clonal type I. Isolation of this genotype from cases of congenital toxoplasmosis has been described, but at a significantly lower rate than type II (Howe & Sibley, 1995), as results of research conducted in France have shown, where out of 86 genotyped isolates only 4 belonged to type I (Ajzenberg et al., 2002).

Sample number	Sample type	Clinical entity	Genotype
1	blood	toxoplasmosis in pregnancy	II
2	amniotic fluid	congenital toxoplasmosis	II
3	blood	congenital toxoplasmosis	I
4*	blood	bone marrow transplantation	II
5*	bronchoalveolar lavage fluid	bone marrow transplantation	II

*samples 4 and 5 are from the same patient

Table 3. Genotypes of human *T. gondii* isolates from clinical samples in Serbia

We have also genotyped isolates from both a blood and BAL sample from an immunosuppressed patient after bone marrow transplantation, which were found to belong to type II. In another study, genotyping of strains isolated from immunosuppressed patients, HIV infected or patients who had undergone organ transplantation, has shown predominance of type II in patients who were infected in Europe (Ajzenberg et al., 2009). On the other hand, isolates that do not belong to this type usually come from people who are infected with *T. gondii* out of Europe. In this group of patients type III was the second in abundance whereas type I was rare (Ajzenberg et al., 2009). In other studies carried out in immunosuppressed patients (patients with AIDS, lymphoma or patients with transplants), which mainly came from France, it was shown that type II isolates were also predominant, while types I and III were isolated rarely (Howe et al., 1997; Honore et al., 2000).

Furthermore, results of a study performed in the USA, based on genotyping of strains isolated from cerebrospinal fluid originating from eight HIV-positive patients showed that most of them were infected with type I strain or strains that have type I alleles (Khan et al., 2005). Although the possible association between clinical entities induced by *T. gondii* with specific *T. gondii* genotypes is yet unclear, it is likely that the resistance or susceptibility to a particular type, especially in immunosuppressed patients, is primarily dependent on individual factors (Ajzenberg et al., 2009). The greatest limitation in genotyping of isolates from clinical samples is the small number of parasites in original material; hence the amount of extracted *T. gondii* DNA is often also small. This problem can be partially eliminated by enriching the sample by bioassay or cell culture, but even the most sensitive molecular methods, such as a multiplex nested PCR, have a threshold of 50 and 25 parasites/mL, respectively (Khan et al., 2005; Nowakowska et al., 2006). The PCR-RFLP protocol by which genotyping was performed in our study has a sensitivity of approximately 170 parasites/mL, which is probably the major reason for the small number of successful genotypizations.

Numerous studies of the *T. gondii* population structure were based on genotyping using a single marker, mostly SAG2 (Howe et al., 1997; Fuentes et al., 2001; Sabaj et al., 2010) and particularly, due to its polymorphisms and sensitivity, GRA6 (Fazaeli et al., 2000; Messaritakis et al., 2008). However, genotyping with a single marker does not allow identification of nonclonal strains, and to determine more precisely the presence of polymorphisms in the population, application of multilocus PCR-RFLP and microsatellite analysis of multiple markers is necessary (Ajzenberg et al., 2005; Su et al., 2006). Although in our experience the GRA6 gene was, due to a small amount of *T. gondii* DNA, the only amplified marker in a blood sample of a neonate suspected of congenital toxoplasmosis (Table 3), that clearly indicated the presence of type I, in our laboratory genotyping is regularly performed using SAG1, SAG2, GRA6 and GRA7 as markers (Miller et al., 2004; Dubey et al., 2007; Prestrud et al., 2008; Richomme et al., 2009; Aubert et al., 2010).

But even the use of multiple markers does not always provide satisfactory results, mainly due to insufficient amounts of extracted parasite DNA. Therefore, there are cases when amplification of all markers in each sample is not successful, as it can be observed in studies performed in the United States and Poland, where PCR-RFLP analysis was carried out also

using four genetic markers SAG2, SAG3, BTUB and GRA6 (Khan et al., 2005; Nowakowska et al., 2006). Using these genetic markers, it was possible to discriminate types I, II and III, but also strains that have a genotype with two allele types at the same locus. Such was the case with one sample in our study which, after the digestion of the product of the amplified GRA7 gene, turned out to possess alleles of both types I and II (Fig. 1, *Mbo* II and *Eco* RI).

Although PCR-RFLP has a limited ability to distinguish between closely related isolates within a clonal line as compared to microsatellite analysis, analysis of up to 9 or 10 genetic markers by this method has been successfully performed in world-class laboratories (Su et al., 2006; Dubey & Su, 2009). On the other hand, the microsatellite analysis is presumed to be more informative to distinguish recent mutations in closely related isolates of the same line, while the RFLP markers are better for detection of time period when the separation of distinct strains in different clonal group has occurred (Su et al., 2006). Multilocus PCR-RFLP genotyping is still the first method of choice in clinical research, mainly for its simplicity and favorable reagent prices, but the best approach for successful genotyping is the use of both methods.

Marker	SAG 1	SAG 2		GRA 6	GRA 7		
Restriction enzyme	<i>Dde</i> I	<i>Mbo</i> I	<i>Hha</i> I	<i>Tru</i> I	<i>Mbo</i> II	<i>Eco</i> RI	<i>Bse</i> GI

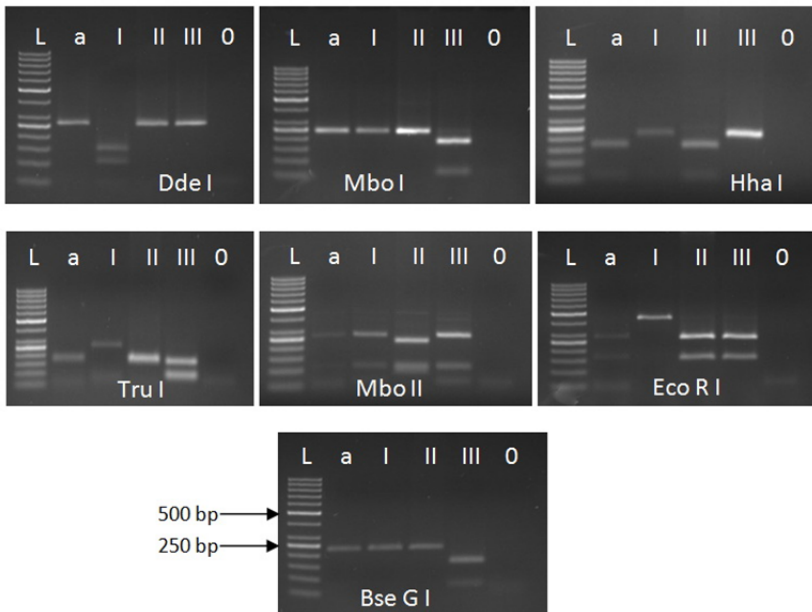


Figure 1. Genotyping pattern summary (markers and restriction enzymes used in genotyping protocol) – illustrative example

Along with the phylogenetic study of *T. gondii*, there is ongoing research aimed at explaining the possible link between the different genotypes and clinical forms of the disease. In spite of the results indicating lack of connection, or a much more complex one than some studies show, there are reported findings on population structure of *T. gondii* that are likely to have important clinical implications. Although it is generally accepted that type II is predominant in cases of congenital toxoplasmosis, at least in Europe and North America (Howe & Sibley, 1995; Howe et al., 1997; Ajzenberg et al., 2002; Darde et al., 2007), type I strains may also be associated with some severe forms of the disease (Howe et al., 1997; Fuentes et al., 2001). Furthermore, strains of atypical genotypes were isolated from immunocompetent patients with severe acquired toxoplasmosis in French Guiana (Carme et al., 2002; Demar et al., 2011), whereas type I and some recombinant strains were isolated from immunocompetent individuals suffering from severe or atypical ocular toxoplasmosis in United States (Grigg et al., 2001).

Even the generally accepted concept of major clinical importance that immunized mothers are resistant to reinfection thereby preventing infection of the offspring, have been recently challenged by insight into the strain variation at the genotype level. Six cases of reinfection among chronically infected pregnant women resulting in a vertical transmission and congenital infection either with a distinct typical or atypical strain have already been reported (Lindsay & Dubey, 2011).

Despite this significant new knowledge, the clinical relevance of the infecting genotypes is an issue that will continue to intrigue researchers in the coming years. Insight into the global population structure of *T. gondii* and its clinical implications, complicated by the growing rate of human migrations among continents, will require wide research efforts based on more standardized protocols, and should include not only clinically manifest cases, but also individuals with asymptomatic infection.

4. Conclusion

The introduction of highly sensitive molecular methods into the diagnosis of toxoplasmosis is of great importance and this paper emphasizes its practical importance and potential as a part of the standard laboratory protocols. Nevertheless, it can be concluded that, at the moment, the best diagnostic approach is a combination of both conventional and molecular methods.

We also present the very first and original phylogenetic data on the *T. gondii* population structure in Serbia. It is shown that in this area, as much as in the rest of the Europe, a clonal population structure is characterized by the predominance of genotype II and much less of genotype I. However, given the fact that the whole region of the Balkan Peninsula is an area of contact with Asia and Africa, where the *T. gondii* population structure is rather different, one may expect a larger diversity, including the presence of clonal type III or even atypical strains, particularly in wild animals.

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Acknowledgement

The work was supported by a grant (project No. III41019) from the Ministry of Education and Science of Serbia.

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