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Evaluation of detection of *Toxoplasma gondii* DNA in animal blood samples by quantitative PCR

Research Article

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Abstract: Aim of the study: The purpose of this study was to find out the relationship between the phase of infection (acute or persistent) and the ability of quantitative PCR to detect DNA of *Toxoplasma gondii* in circulating leukocytes in blood. Methodology: Animal serum samples were examined (50 sheep, 47 dogs, 32 dairy cows, 91 wild boars and 36 rabbits) for the occurrence of IgM and IgG antibodies to *T. gondii* by ELISA. Uncoagulated blood samples from the same animals were examined for the detection of *T. gondii* DNA in circulating leukocytes by real-time PCR. Results: Only IgM antibodies, characteristic for acute infection, were detected in 45 of the 256 serum samples (17.6%). Only IgG antibodies, corresponding with chronic infection, were detected in 120 of the 256 samples (46.8%). In 91 of the 256 samples (35.5%) neither IgM or IgG were detected by ELISA. For real-time PCR, animals were divided into three groups based on the serological results : (group I - acute infection, group II - chronic infection, and group III - no infection). In group I, the presence of *T. gondii* DNA was detected in 9 out of 45 samples (20%), whereas in group II only 1 of 120 samples was positive for *T. gondii* DNA (0.8%). In group III, no DNA of *T. gondii* (0/91 samples) was detected by real-time PCR. Significance: The proof of DNA by real-time PCR in IgM positive samples was statistically significant in comparison to IgG positive samples ($P<0.0001$).

Keywords: *Tosoxoplasma gondii* • *Toxoplasmosis* • *Molecular method* • *Serological method*

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1. Introduction

The microorganism *Toxoplasma gondii* infects wild and domestic animals including birds, cats, sheep, goats, cattle and pigs. Cats are the most common source of the Toxoplasma protozoa that are transmitted to other animals or people. To this day, animals and animal products represent an important source of toxoplasmosis infection for humans. Epidemiologically, the transmission of *T.gondii* is possible by ingesting infectious oocysts from the environment. Tachyzoites can be transmitted by contaminated blood products, tissue transplants and the drinking of unpasteurised milk. But the most important source of infection is bradyzoites contained in meat or primary offal of many different animals [1,2].

Serological diagnosis of active infections is unreliable because reactivation is not always accompanied by changes in the antibody levels. In many cases when the chronic infection is reactivated into acute infection (e.g. in immunosuppressed patients - AIDS, after transplantation), this reactivation is not related to the increase of IgM antibodies which are characteristic for the acute infection. Thanks to more sensitive new serological methods, persistence of immunoglobulin M (IgM) antibodies during the chronic stage of infection was observed, thus complicating the interpretation of serological results. In addition, conventional techniques to determine the presence of antibodies may fail to detect specific anti-Toxoplasma immunoglobulin G (IgG) or IgM during the active phase of infection, e.g. in cases

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of immunosuppressive therapy in allogeneic bone marrow transplant recipients and in patients with acquired immunodeficiency syndrome (AIDS; [3]). Molecular tests that could detect the presence of circulating parasites could be useful in such situations. A positive serological result is only indicative of infection, whereas direct detection of *T. gondii* in blood or other clinical samples categorically confirms the parasite presence leading to the diagnosis of primary, reactivated or chronic toxoplasmosis [4].

2. Experimental Procedures

The study analyzed 256 blood samples collected from different animals including 50 sheep, 47 dogs, 32 dairy cows, 91 wild boars and 36 rabbits.

The blood samples were taken from *vena jugularis* in the dairy cows and sheep and from *vena cephalica* in dogs. Blood samples from wild boars and rabbits were taken after they were hunted. Blood samples (5 ml) were collected to tubes without anticoagulant and after coagulation at 22°C for 30 minutes and subsequent centrifugation (2200x*g*, 10 minutes), the sera samples were stored at -20°C until used. Blood was also collected in tubes containing EDTA as an anticoagulant. These uncoagulated blood samples were used for DNA extraction. DNA was isolated from the leukocyte fraction of uncoagulated blood after separation by centrifugation at 4000x*g* for 15 minutes at room temperature. Total DNA was then purified from white blood cells by using the commercial kit QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer´s instructions. The DNA samples were frozen after the isolation and stored at a temperature of -18°C until analysis.

2.1 Serological analysis

An enzyme-linked immunosorbent assay (ELISA) was carried out for the detection of IgG and IgM antibodies to *T. gondii* according to the manufacturer´s instructions (Test-Line, Czech Republic). In the first step, specific IgG or IgM antibodies in serum were bound to the *T. gondii* antigen coated on the surface of reagent wells and then, the rabbit anti-species IgG or IgM antibodies (sheep, dog, rabbit, cattle, and wild boar) labelled with peroxidase (Sigma- Aldrich, USA) were applied to the complex formed between the *T. gondii* antigen and circulating antibodies. After addition of the enzyme substrate, TMB (3,3´,5,5´-tetramethylbenzidine), the absorbance was read at 450 nm using a Dynex spectrophotometer (Dynex Technologies, USA). Positive and negative serum controls previously tested by conventional serological test were included on each plate. For each sample, the index of positivity (IP) was calculated according to the schema provided by the manufacturer: IP= sample absorbance/average absorbance of cut-off serum (cut-off serum is a serum sample which contains antibodies to *T. gondii* in limiting concentration). Samples with IP<0.8 were considered to be negative, samples with IP between 0.8-1.0 were considered to be dubious and samples with IP>1.0 were considered to be positive [5].

2.2 Quantitative real-time PCR

Amplification of the isolated DNA was carried out by the real time PCR with SYBR green as a detection system from the *T. gondii* gene region TGR1E, repeated in the genome 30-35 times, using the specific primers TGR1E-1 and TGR1E-2 [6]. Cloned *T. gondii* TGR gene (GenExpress, Germany) diluted to 10^4-10^9 was used for the calibration curve. In each reaction, a melting analysis (comparison of the melting temperature (Tm) of PCR products) was determined to differentiate specific and non-specific PCR products.

The reaction volume was 25 μl, which contained commercial FastStart Universal SYBR Green Master (Roche, Germany) and 0.2 μM primers (TGR1-1 and TGR1E-2). Real-time PCR was completed using a thermocycler Line GeneK with the software Line GeneK Fluorescent Quantitative Detection system (BIOER Technology, China). After incubation at 50°C for 2 minutes and initial denaturation at 95°C for 10 minutes, 40 amplification cycles were performed (95°C for 15 s, 60°C for 1 minute). Melting analysis was carried out at temperatures ranging from 60°C to 95°C, in which the temperature was gradually increased by 0.5°C and the period of measurement at individual steps was 15 s. Every PCR run included a control without DNA (containing the reaction mix alone and nuclease-free water).

2.3 Groups of animals

Each sample was examined by ELISA for detection of IgM and IgG specific antibodies to *T. gondii*. After serological examination samples were divided into three groups. Group I contained samples only positive to IgM antibodies (acute infection), group II contained samples positive only to IgG antibodies (chronic infection) and, group III contained samples without IgM and IgG antibodies. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

2.4 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 software. Fisher´s exact test was used to

compare the success of real-time PCR depending on the presence of IgM or IgG antibodies.

3. Results

By ELISA, IgM antibodies, which appear at the beginning of infection and which are characteristic for acute infection, were detected in 45 out of 256 samples (17.6%). IgG antibodies, which corresponded to chronic infection, were detected in 120 out of 256 samples (46.8%). In 91 of the 256 (35.5%) animal blood samples neither IgM or IgG were detected by ELISA. The occurrence of IgM or IgG antibodies in each group of animals is summarized in Table 1.

According to the serological results animals were divided into three groups: animals with suspicion of acute (group I, n=45) or chronic toxoplasmosis (group II, n=120) and without infection (group III, n=91). For statistical analysis we compared group I (acute infection, IgM positive) and group II (chronic infection, IgG positive).

Using quantitative real-time PCR the presence of *T. gondii* DNA was detected and the number of copies quantified in the 256 non-coagulated animal blood samples. Using real-time PCR, *T. gondii* DNA was detected and quantified in ten samples of non-coagulated blood (6 sheep, 1 wild boar and 3 rabbits; Table 2). For animals presenting acute toxoplasmosis (group I), the presence of *T. gondii* DNA was detected in 9 out of 45 samples (20%), whereas in the chronic group (group II) only one sample was positive (n=120; 0.8%). In group III, which contained animals without IgM or IgG antibodies, no DNA of *T. gondii* (n=91) was detected by real-time PCR.

The proof of DNA by real time PCR in IgM positive samples was statistically significant in comparison to IgG positive samples ($P<0.0001$).

Standards with the known dilution of *Toxoplasma* DNA were used to determine the detection limit of a modified real-time PCR and to create a calibration curve that ranged from 10^9-10^4 copies of *Toxoplasma* DNA. The correlation coefficient of the calibration curve was 0.998. As SYBR Green a fluorescent dye, was used as a detection system, a melting analysis was a part of the real-time PCR to distinguish between specific and non-specific products. During the melting analysis, the melting temperature (Tm) of a positive control and positive samples was 84°C. In quantifying the examined samples within a 40-cycle protocol for the real-time PCR, the number of copies detected in the positive samples ranged from $1.07x10^2$ to $1.49x10^5$ (Table 3).

Animals	IgM		IgG		Negative
	N/n	%	N/n	%	
Sheep	27/50	54%	23/50	46%	0
Cattle	4/32	12.5%	20/32	62.5%	8
Rabbits	7/36	19.4%	24/36	66.6%	5
Wild boars	5/9	15.5%	18/91	19.8%	68
Dogs	2/47	4.3%	35/47	74.5%	10
Total	25/256	17.6%	120/256	46.9%	91

Table 1. Occurence of IgG and IgM antibodies to *T. gondii* by ELISA in different animal species.

N – number of positive samples; n – number of examined samples

	N	Real-time PCR		P value
		Positive	Negative	
Group I (IgM+)	45	9	36	<0.0001*
Group II (IgG+)	120	1	119	-

Table 2. Relation between the presence of *T. gondii* DNA and serological results.

Group I-acute toxoplasmosis; Group II-chronic toxoplasmosis; P value was obtained by comparison of the proof of Toxoplasma DNA by qPCR in group I (acute infection, IgM positive) and group II (chronic infection, IgG positive).*

4. Discussion

The diagnosis of toxoplasmosis may be established by serological tests, PCR, histological demonstration of the parasite and/or its antigens (e.g. immunoperoxidase stain), or isolation of the organism. Serological tests show the presence of IgM antibodies, which can represent acute infections as well as residual IgM antibodies in chronic infections. Avidity tests may help in this setting by distinguishing between IgG of high and low affinity, corresponding to either chronic or acute toxoplasmosis. Molecular tests detecting circulating parasites would be helpful in the final diagnosis. A positive serological result is only indicative of infection, whereas direct detection of *T. gondii* in blood or other clinical samples categorically confirms the parasite presence in the organism [4,7]. Several indirect methods have been proposed for the detection of antibodies to *Toxoplasma* in animals, generally in samples of serum and plasma. In addition, thoracic fluid of aborted fetuses, milk and samples of fluid obtained by freezing and thawing portions of muscular tissue (meat juice) can be tested for antibodies. There is a wide variety of serological assays available and in use. However, data from different studies may not always be directly comparable due to discrepancies in the procedures used for the detection of antibodies. For example, the modification of protocols, the use of different strains of *Toxoplasma*, and the different cut-off points of tests. Not all assays are suitable for every animal species and cross reactions with antibodies to related parasites may result in false positives. Direct methods, such as PCR, need biopsy samples [8,9]. Methods of sampling of the brain and other internal organ tissues in animals are not as sophisticated as those used in humans. This is particularly true of the large meat-producing animals (e.g. cattle, sheep, goats and pigs), which posethe greatest risk of toxoplasmosis transmission to humans. In our study we decided to use blood as the main sample type for isolation and detection of *T. gondii* DNA. The purpose of this study was to find out the relationship between the phase of infection (acute or persistent) and the ability of quantitative PCR to detect DNA of *T. gondii* in circulating leukocytes in blood. Our study has shown that PCR analysis of animal blood can only detect DNA of *T. gondii* in acute phase of infection. At this time in the infection, parasites are hidden within leukocytes that are circulating in the blood stream. At this stage, we were able to capture parasites and isolate DNA of *T. gondii*. After initiation of the chronic phase, parasites are hidden within cysts in the tissues and organs of the animal and it is not possible to detect their presence in the blood. DNA of *T. gondii* was confirmed in 10 animals from the total of 256 animals sampled. Of the ten PCR-positive animals, DNA was detected in nine individuals with ongoing acute phase of infection (confirmed by ELISA). Hitt and Filice [10] detected *T. gondii* DNA in 12 out of 32 (37%) rabbit blood samples by PCR. The decreased PCR sensitivity in blood samples was believed to be influenced by localization of leukocytes. In their study leukocytes in heparinised blood were not localized in the leukocyte layer but they were distributed widely, mostly in the erythrocyte layer. Therefore,the choice of the genome area which is amplified is important for efficiency of PCR analysis. Hitt and Filice believed that the B1 gene enhanced the sensitivity of PCR techniques from blood samples [10]. Other investigators have been unable to detect *T. gondii* DNA in bone marrow from humans or whole blood from mice with toxoplasmosis. Heme, heparin, and other poorly characterized substances have been reported to decrease sensitivity [11]. Kompalic-Cristo *et al.* examined 183 buffy coat samples from serologically examined patients, of the IgM seropositive patients 48.6% presented parasiteamia proven by PCR, whereas only 3.6% positivity was achieved in individuals with chronic infection [12]. Lamoril *et al.* examined 19 patients with confirmed cerebral toxoplasmosis and in only three cases samples were PCR positive. In the case of generalized toxoplasmosis, the lymph nodes, liver and spleen could be affected by infection and in this situation there is higher possibility to detect *Toxoplasma* DNA by PCR [13]. Truppel *et al.* examined Capybaras, *Hydrochaeris hydrochaeris*, by serological test and also examined lymph node, liver, spleen, heart and blood samples for the detection of *Toxoplasma*

Sample	Number of copies	Group
Sheep 1	$5.92x10^4$	IgM+
Lamb 1	$1.49x10^5$	IgM+
Sheep 3	$3.67x10^4$	IgM+
Sheep 4	$5.75x10^2$	IgM+
Sheep 5	$3.89x10^4$	IgM+
Sheep 6	$2.56x10^3$	IgM+
Wild boar	$1.05x10^5$	IgM-
Rabbit 1	$1.07x10^2$	IgM+
Rabbit 2	$2.09x10^2$	IgM+
Rabbit 3	$3.17x10^2$	IgM+

Table 3. The number of *T. gondii* DNA copies in the examined samples in a 25 μl-volume.

DNA. *Toxoplasma* DNA has been detected both in the liver and in the blood [14].

In general PCR techniques are less sensitive in the diagnosis of toxoplasmosis. One of the main problems is missing standardization of PCR performance according to laboratory conditions. The other problem is the kind of tested tissue (e.g. blood, liver, spleen, cerebrospinal fluid). Each study has different sensitivity in the PCR with different tissues. Comparison studies, that compare PCR using different tissue samples, give us the better view on sensitivity of PCR and help us to choose the best tissue samples with regard to ease and the least invasive for the animal.

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