Research Article

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Seroprevalence of *Toxoplasma gondii* infection in goats from the south-west region of Poland and the detection of *T. gondii* DNA in goat milk

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Abstract: *Toxoplasma gondii* (Nicolle et Manceaux, 1908) is an obligatory intracellular protozoan parasite prevalent in animals and humans worldwide having medical and veterinary importance on account of causing abortion or congenital disease in intermediate hosts, including man. Since *T. gondii* has already been identified in the milk of goats, *Capra aegagrus hircus* (Linnaeus), the possibility of acquiring infection by ingesting unpasteurised goat milk should be taken into consideration. Thus, the aim of the present study was to determine the presence of *T. gondii* DNA in goat milk. First, 73 goats (females) from 36 farms located in Poland were examined serologically by direct agglutination test (DAT) to estimate the *T. gondii* serological status. Milk samples from 60 selected lactating females were examined for the presence of *T. gondii* DNA by Real time PCR and nested PCR (B1 gene). To estimate the clonal type of detected *T. gondii*, multiplex PCR was performed using 6 markers. In DAT, positive results were found in 70% of 73 goats. Among examined 60 milk samples, 65% were positive in Real time PCR and 43% in nested PCR. It is noteworthy that 11 samples positive in PCR were collected from seronegative goats. The multilocus PCR analysis mostly revealed the occurrence of genotype III, which is relatively rare in Europe. The recorded high prevalence of anti-*Toxoplasma* antibodies in tested goats (70%), associated with a high prevalence of *T. gondii* DNA in goat milk samples (65%), indicates a potential risk of the parasite transmission through goat milk ingestion.

Keywords: toxoplasmosis, DAT, PCR, genotyping, serology

Toxoplasma gondii (Nicolle et Manceaux, 1908) is an obligatory intracellular protozoan parasite that causes infection in humans and in many species of wild and domestic animals. Infection in humans with *T. gondii* may pose a severe medical problem in a congenital form, causing cerebral and ocular damage in newborns, and as an acquired infection in immunocompromised individuals (Dubey and Beattie 1988). Humans usually become infected by ingestion of undercooked meat containing tissue cysts, or by consumption of food or water contaminated by oocysts excreted by cats.

Tachyzoites of *T. gondii* have already been identified and isolated from the milk of goats, *Capra aegagrus hircus* (Linnaeus), and infections of goats have been noted worldwide. Therefore, the possibility of acquiring infection by ingesting unpasteurised goat milk should be taken into consideration (Mancianti et al. 2013, Dubey et al. 2014). In fact, cases of clinical toxoplasmosis have been reported in humans after drinking unpasteurised goat's milk (Dubey et al. 2014). Fresh dairy products made from raw goat milk

could be also a potential source of infection with *T. gondii* (see Walsh et al. 1999).

The aim of the present study was to determine the presence of *T. gondii* DNA in milk samples of goats from Poland.

MATERIALS AND METHODS

Animals. The examined goats originated from the south-western part of Poland, the traditional region of goat breeding. In total, 73 females aged of 0.5–20 years (mean = 4.6, SD = 3.9) from 36 farms were examined serologically to estimate serological status.

Serological examination. Blood was taken from the goats by jugular punctures and allowed to clot at room temperature. After centrifugation, serum was stored at -20 °C until testing. Antibodies against *T. gondii* were detected by commercial direct agglutination test (DAT) (Toxo-Screen DA, bioMérieux, Marcy-L'Étoile, France) in accordance with manufacturer's instructions. A titre of 1 : 40 was considered as evidence of exposure to *T. gondii*.

Goat milk examination. Milk samples (4 ml) were collected from 60 lactating females by manual milking of teats and stored

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frozen. Before analysis, the milk samples were centrifuged, and to avoid interference by casein, pellet of milk was treated with 200 µl TE (1 mM EDTA, 10 mM Tris-HCl (pH = 7.6) (Sigma-Aldrich Co., St. Louis, USA) and 300 µl 0.5 M EDTA (pH 8) (Sigma-Aldrich), resuspended and centrifuged at 3,000 g for 10 min. Next, the milk samples were diluted in 200 µl of PBS, according to the method described by Psifidi et al. (2010).

DNA from milk samples was extracted using QIAamp DNA mini kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instruction. Real time PCR was performed according to the method described by Lin et al. (2000) using commercial master mix QI Supermix (Bio-Rad, Hercules, California, USA). The amplification was carried out in a thermal cycler CFX-96 (Bio-Rad).

Detection of the DNA of *T. gondii* was performed by amplification of 35-fold-repetitive *B1* fragment gene in nested PCR, according to the method by Grigg and Boothroyd (2001). To estimate the clonal type for *B1* gene positive samples, multilocus PCR was performed using markers SAG1, SAG2 (5'-SAG2 and 3'-SAG2), SAG3 and GRA6 according to method described by Su et al. (2010). Nested PCR and Multilocus PCR were carried

out in a C1000 Thermal Cycler (Bio-Rad). In each PCR tests, as positive controls isolates of the DNA of *T. gondii* of RH (type I), ME49 (type II) and C56 (type III) strains were used, nuclease-free water was used as the negative control.

DNA sequencing of amplicons was performed using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA, USA), with the use of Abi Prism Big Dye Terminator v. 3.1. Cycle Sequencing Kits and Big Dye X Terminator Purification Kit (Applied Biosystems). Sequences were analysed using BioEdit and MEGA 7 software, and compared with the NCBI database using Blast.

The results were analysed with χ^2 test, using STATISTICA v. 5.1 package (Statsoft, Tulsa, OK, USA).

RESULTS

The positive results in DAT were found in 51 of 73 (70%) tested goats (Table 1). Among positive results 21.5%, 17.5% and 61% were detected in titre ranges 40–180, 540–4,000 and $\geq 6,000$, respectively.

Among the examined 60 milk samples, 39 (65%) were positive in Real time PCR and 26 (43%) in nested PCR. It

Table 1. Summary of direct agglutination test (DAT) and PCR results for *Toxoplasma gondii* in goats.

Farm No.	No. of animals	Age (years)	Results (No. of positive/No. of examined)		
			DAT (titre)	Real time PCR	Nested PCR (B1)
1	2	5; 3	6,000; 0	0; 1	0; 1
2	3	5; 2; 2	0; 0; 18,000	1; 1; 1	0; 0; 0
3	2	6; 3	18,000; 0	1; 0	0; 0
4	2	3; 3	6,000; 6,000	0; 1	0; 0
5	2	3; 1	0; 0	n/e; 1	n/e; 1
6	1	3	54,000	0	0
7	3	3; 3; 5	0; 0; 18,000	1; 1; 0	0; 0; 0
8	1	8	4,000	1	1
9	2	3; 3	6,000; 54,000	1; 1	0; 1
10	1	5	180	0	0
11	3	5; 6; 2	40; 18,000; 18,000	0; 0; 1	0; 0; 1
12	1	6	180	1	0
13	1	15	0	1	1
14	1	2	0	0	0
15	1	16	18,000	1	1
16	3	5; 4; 2	4,000; 40; 0	1; n/e; n/e	1; n/e; n/e
17	1	1.5	0	n/e	n/e
18	6	5; 3; 2; 1; 1; 1	6,000; 18,000; 54,000; 6,000; 4,000; 6,000	0; 0; 1; 0; 1; 1	0; 1; 1; 0; 1; 0
19	1	5	6,000	1	1
20	2	2; 3	0; 6,000	1; n/e	1; n/e
21	3	13; 2; 1	6,000; 180; 4,000	0; 1; 1	1; 1; 1
22	5	4; 2; 10; 5; 5	4,000; 0; 6,000; 180; 4,000	0; n/e; 1; 1; 1	0; n/e; 1; 1; 0
23	2	1; 3	540; 54,000	1; 0	0; 0
24	1	10	6,000	1	0
25	1	5	54,000	1	0
26	2	0.5; 0.5	0; 0	n/e; n/e	n/e; n/e
27	1	0.5	0	n/e	n/e
28	2	1; 7	0; 40	1; 0	0; 1
29	1	1	6,000	0	0
30	3	4; 14; 5	0; 6,000; 54,000	1; 1; 1	0; 0; 1
31	3	10; 10; 10	40; 4000; 6,000	0; 1; n/e	0; 1; n/e
32	1	20	6,000	0	1
33	2	6; 5	18,000; 18,000	0; 1	0; 1
34	4	0.5; 0.5; 6; 6	0; 0; 180; 0	n/e; n/e; 1; 1	n/e; n/e; 1; 1
35	2	4; 0.5	1,620; 40	0; n/e	0; n/e
36	1	8	40	1	1
Total	73	4.6 (mean); 3.9 (SD)	51/73 (70%)	39/60 (65%)	26/60 (43%)

n/e – not examined – no milk samples available.

Table 2. Results of genotyping of *Toxoplasma gondii* detected in goat milk.

Total No. of milk samples	SAG1	3'SAG2	5'SAG2	SAG3	BTUB	GRA6
1	n/a	I/III	III	III	n/a	III
2	n/a	I/III	III	n/a	I/III*	n/a
2	n/a	I/III	III	n/a	n/a	n/a
1	II/III#	n/a	n/a	III	n/a	n/a
1	n/a	n/a	III	n/a	n/a	III
1	II/III**	n/a	n/a	n/a	n/a	n/a
3	n/a	I/III	n/a	n/a	n/a	n/a
6	n/a	n/a	III	n/a	n/a	n/a
2	n/a	n/a	n/a	III	n/a	n/a
6	n/a	n/a	n/a	n/a	n/a	III***

n/a – product not amplified; I–III – clonal lineage; GenBank accession numbers: * KY634427, KY634428; ** KY634429; *** KY634421–KY634426; # KY634430.

is noteworthy that 11 samples positive in PCR were collected from seronegative goats.

Analysis of genotyping showed that amplification in used markers (SAG1, 3'SAG2, 5'SAG2, SAG3, BTUB and GRA6) was possible for selected *Bl* gene-positive sample. Two samples were positive in four and three markers, another four samples amplified in two markers. For the remaining 17 samples, the positive reaction was obtained only in one marker (Table 1). No new single-nucleotide polymorphisms (SNPs) were found. For 14 samples only type III lineage was determined. Eleven samples had alleles type III at the 5'SAG2 locus, four samples at the SAG3 locus and seven samples at the GRA6 locus. Seven samples had alleles types I/III at the 3'SAG2 locus and one at the BTUB locus. Two samples had alleles II/III types at the SAG1 locus (Table 2).

DISCUSSION

The present study showed 71% of seropositive results among the tested goats, this is at the upper limit of the results from various European countries. The results obtained in the present study are meaningful not only because of high seropositivity, but also because of a very high proportion (61%) of strong reactions with titres 6,000 and higher. High seropositivity reported in goats worldwide (Iovu et al. 2012, Djokic et al. 2014) indicates that goats appear to be susceptible to *T. gondii* infection. Clinical toxoplasmosis with an abortion and neonatal mortality, as well as fatal cases of acute toxoplasmosis in goats were also reported (Dubey and Beattie 1988, Masala et al. 2007, Mancianti et al. 2013). In Poland, about 30–100% of goats are exposed on *T. gondii* (Michalski and Platt-Samoraj 2004, Czopowicz et al. 2011). The examined goats were most often kept in backyard systems, less frequently in large specialised herds or for hobby purposes. Goats were usually stabled together with other animals, including cats. Thus, contaminated pasture and water seem to be the main sources of infection with *T. gondii*, which was also indicated by others authors (Deng et al. 2016).

In the present study, the DNA of *T. gondii* was found by PCR in 43–65% of goat milk samples. The positive PCR results were found also for 11 milk samples from seronegative goats, which can be explained by the initial phase of infection and undetectable level of specific antibodies. The PCR results in the present study are much higher than recently reported in Poland (3%) by Cisak et al. (2017). A lower percentage (6%) was obtained in Brazil (Bezerra et al. 2015) and Italy (13%) (Mancianti et al. 2013). A relatively high prevalence (33%) was recorded by Spišák et al. (2010) in Slovakia. The presence of DNA of *T. gondii* in goat milk is not equivalent with the viability of the parasite, but drinking unpasteurised goat milk was found to be a risk factor for acquired toxoplasmosis in pregnant women in Europe (Cook et al. 2000) and in the United States (Dubey et al. 2014). Moreover, the results of experiments by Dubey et al. (2014) showed that *T. gondii* can be excreted intermittently in goat's milk, and the parasite can also survive in cheese made from unpasteurised, cold goat's milk.

In the present study, bioassay was not performed and none of viable tachyzoites were isolated from the milk samples. Therefore, it is possible that tachyzoites in milk (all or in part) could be inactivated among others by specific antibodies whose high level (titre 6,000 and higher) at the large number of examined animals was found. However, the presence of viable tachyzoites of *T. gondii* in the milk of chronically infected goats can be due to possible resurgence of tissue with cysts tachyzoites of *T. gondii* which can be excreted in the milk during physiological decrease in the peripartum immunity (Silva et al. 2010). Since goat milk and milk product are often used unpasteurised, it should be taken into consideration that the presence of the DNA of *T. gondii* in milk samples can also increase the possibility of live parasite transmission.

Most of strains of *T. gondii* detected in humans and animals in Europe are one of three types of clonal lineages (I, II or III), but atypical strains were also found (Ajzenberg 2010). Type II strains are the most common in chronic infection, whereas type III strains are responsible for infection primarily in domestic animals. In France, type II was found in more than 90% of human congenital toxoplasmosis, but also in all isolates originating from a large number of animal species (Ajzenberg et al. 2002, Dardé 2008). However, studies in Portugal, Spain and the Slovak Republic indicate that type III or type I may be more frequent in these countries (Fuentes et al. 2001, de Sousa et al. 2006, Turčková et al. 2013). Moreover, Berger-Schoch et al. (2011) reported that sheep in Switzerland were infected with *T. gondii* with clonal type II, but cattle were infected with the clonal types I or III, or atypical *T. gondii*. RFLP analyses of SAG2 locus carried on protected wildlife in the Tatra National Park, Slovakia, revealed that 44% of isolates of *T. gondii* were characterised as genotype III, 39% were represented by genotype II and 17% isolates belonged to genotype I (Turčková et al. 2014).

In our survey we have confirmed mainly genotype III of *T. gondii* in genotyped samples. However, it was not possi-

ble to distinguish types I and III or II and III (Table 2). These results can also indicate a possible combination of different alleles, not type III alone. Genotyping by one marker only (possible for the majority of samples) could also be insufficient for definitive analysis. Unsuccessful PCR amplification for all six tested markers may be due, at least in part, to the low amount of DNA, which is not quite suitable for genotyping. Data concerning typing of the DNA of *T. gondii* isolated from goat milk are scarce. In another study, by Mancianti et al. (2013) among ten PCR positive samples, genetic characterisation revealed the occurrence of genotype III from seven samples, genotype I from one sample, and atypical genotypes from two samples.

The present study focused on the assessment of potential risk associated with the ingestion of goat milk. To the best of our knowledge, this is the first report on the genetic characterisation of *T. gondii* detected in goat milk in Poland.

Considering the fact that goats can represent an important source of milk, they seem to play a very important role

in the epidemiology of human toxoplasmosis. In order to decrease the risk by avoiding ingestion of raw goat milk, appropriate health education is needed worldwide.

The recorded high prevalence of anti-*Toxoplasma* antibodies in goat blood (70%), with a high percentage of titre 6,000 and above, associated with a high prevalence of *T. gondii* DNA in goat milk (65%), indicates a potential risk of the transmission of *Toxoplasma* through raw milk ingestion. Current consumer recommendations to avoid drinking raw milk, or eating fresh dairy products made from raw milk seems to be accurate in the light of the findings of the present study. Obtained data can also be useful for farmers and veterinarians, to improve the methods of management and prevention on goat farms.

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