

SHORT REPORT

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Detection and genotyping of *Toxoplasma gondii* DNA in the blood and milk of naturally infected donkeys (*Equus asinus*)

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Abstract

Background: *Toxoplasma gondii* is a worldwide zoonotic protozoan. Consumption of raw milk from infected animals is considered a risk factor for acquiring toxoplasmosis in humans. Recently, donkey milk has been indicated for therapeutic and nutritional purposes and *T. gondii* infection is common in donkeys. The purpose of the present paper was to detect the presence of parasite DNA in milk of *T. gondii* positive donkeys.

Findings: Antibodies to *T. gondii* were found in 11 out of 44 healthy lactating donkeys by IFAT. *T. gondii* DNA was detected by PCR in blood of 6 and milk of 3 seropositive jennies. Results of limited RFLP-PCR genotyping indicated the presence of *T. gondii* genotype II or III, commonly found in Europe.

Conclusions: The occurrence of *T. gondii* DNA in milk suggests that the consumption of raw milk from seropositive donkeys could be a potential source of human infection.

Keywords: *Toxoplasma gondii*, Donkey, Seroprevalence, Milk, Genotype, Italy

Findings

Infections with *Toxoplasma gondii* are widely prevalent in humans and animals, especially food animals throughout the world [1]. Infections are usually asymptomatic in immunocompetent individuals but vertical transmission in humans can lead to the risk of stillbirth, fetal death in utero, or severe central nervous system involvement in newborns, such as cerebral calcifications and hydrocephalus [2]. In immunocompromised individuals toxoplasmosis may cause encephalitis, pneumonitis and life-threatening disease [3]. Drinking raw goat milk has been identified as one of the risk factors for acquiring postnatal toxoplasmosis in humans and pigs [1].

During the last five decades, Italian autochthonous donkeys (*Equus asinus*) suffered from a severe reduction in population size, which led to the extinction of six breeds [4]. At present, nine breeds remain in this country, all classified by FAO as critically endangered or endangered. These include the Asino Amiatina, Asino Argentato di Sologno, Asino Asinara, Asino di Martina Franca, Asino di

Pantelleria, Asino Ragusana, Asino Romagnolo, Asino Sardo, and Asino Grigio Siciliano [4]. As a consequence, Italian public institutions and private breeders associations made strong efforts to preserve the still existing autochthonous breeds [5]. Following this strategy, efforts are being made to promote the use of all the remaining Italian donkey breeds in recreational activities (agritourism, trekking) and onotherapy (especially with children) as well as in meat (salamis, stew) and milk (cosmetics industry, human nutrition) production [4-6]. The composition of donkey milk is the closest to woman's milk (relatively poor in protein and fat but rich in lactose) and can be used as a possible substitute for babies, children and adults with Ig E-mediated cow milk allergy [7]. Donkey milk is also recommended as an aid in the prevention of atherosclerosis [8] and tumor therapy [9].

In Italy, farmers are required to follow good hygienic practices but there are no specific rules for donkey breeding and milk production [6]. According to the existing European legislation (reg. EC 853/ 2004 and 1662/2006), raw milk from any species can be sold immediately after milking and directly by the producer to the consumer, or to a local milk seller which in turn is the supplier to final

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Table 1 *Toxoplasma gondii*-antibody titers and results of molecular analysis as determined by immunofluorescence antibody test (IFAT) and nested-PCR (n-PCR) performed on serum, blood and milk samples of lactating jennies, respectively

Sample ID	IFAT titers	Blood n-PCR	Milk n-PCR
1	1/40	Positive	Positive
2	1/160	Positive	Positive
3	1/40	Positive	Positive
4	1/160	Positive	Negative
5	1/80	Negative	Not performed
6	1/20	Negative	Not performed
7	1/20	Negative	Not performed
8	1/20	Positive	Negative
9	1/20	Negative	Not performed
10	1/40	Positive	Negative
11	1/20	Negative	Not performed

consumers, without any thermal treatment except refrigeration between 0 and 4°C [10]. Given that, recently donkey milk has been increasingly rediscovered as an appreciated source of nourishing food for humans [6], knowledge on safety, quality and hygiene of this milk may become of crucial importance to some categories of consumers (cases of low immune system defenses, elderly, convalescence, infants with cow milk allergy when breast-feeding is not possible). The quality of raw donkey milk has been evaluated microbiologically [11]. Recently, viable *T. gondii* has been detected in raw milk from cows, sheep, goats, buffaloes, and camels [12]. The aim of the present study was to detect *T. gondii* in donkey milk.

In the present study, blood and milk specimens from 44 adult lactating jennies (Asino Amiatina breed, 6 to 14 years old) were obtained during winter 2013. The animals were semi-intensively farmed in paddocks and were healthy, as confirmed by general physical examination. The Asino Amiatina breed was chosen arbitrarily since Tuscany is the top region in terms of population of these donkeys in Italy [4]. Antibodies to *T. gondii* were assayed by immunofluorescent antibody test (IFAT), using commercially

available antigen coated 12 well slides (VMRD Inc., Pullman, Washington, USA) and anti-horse-IgG FITC antibody produced in rabbit (Sigma-Aldrich; PBS dilution 1:32). All serum samples were screened at a dilution of 1:20, and positive sera were end-titrated using 2-fold dilutions. After results of serological tests were known, blood samples from seropositive jennies were processed for DNA extraction and subsequent amplification by nested-PCR (n-PCR) as previously described [13], while samples from seronegative jennies were discarded. Similarly, when results on blood samples were known, milk samples (50 ml) from n-PCR positive jennies were processed as above, while samples from negative jennies were discarded. Milk sampling was performed under sterile condition; teats were cleaned and wiped, and 3 squirts of milk were discarded prior to collection in sterile single use plastic vials. Milk contains minor quantities of nucleated cells in comparison to whole blood, so prior to DNA extraction, concentration was carried out by centrifugation at 2200 g for 5 minutes [14]. To avoid interference by casein, 1 ml of pellet was treated with 200 µl TE [1 mM EDTA, 10 mM Tris-HCl (pH = 7.6)] and 300 µl 0.5 M EDTA (pH = 8), then it was resuspended and centrifuged at 3000 g for 10' [15]. Somatic cells were diluted in 200 µl of PBS and DNA was extracted from both blood and milk somatic cells using the QIAamp® DNA minikit (Qiagen, Milan, Italy) in accordance with the manufacturer's instructions. The thermic cycle step at 94°C for 5' we used also denatures the lactoperoxidase present in milk; lactoperoxidase can act against the Taq DNA Polymerase in PCR based-methods. Genotypic characterization of *T. gondii* DNA was performed by PCR amplification of 12 genetic markers (SAG1, 3-SAG2, 5-SAG2, SAG2 new, SAG3, BTUB, GRA6, C22-8, C29-2, L358, PK1, and Apico) as reported [16].

Antibodies to *T. gondii* were found in 11 out of 44 donkeys with antibody titers of 1/160 (n = 2), 1/80 (n = 1), 1/40 (n = 3) and 1/20 (n = 5). *T. gondii* DNA was recovered from blood of 6 and milk of 3 seropositive donkeys (aged 8, 11 and 14 years, respectively). Results of IFAT and n-PCR are summarized in Table 1. Results of genotyping are shown in Table 2. Although we did not get amplification with all markers, available data indicated the presence of genotype

Table 2 *Toxoplasma gondii* genotypes identified in blood and milk of PCR positive jennies

Sample identification nos.	Genetic markers											
	SAG1	3'SAG2	5'SAG2	SAG2 new	SAG3	BTUB	C22-8	C29-2	GRA6	L358	PK1	Apico
1	ND	III	III	III	III	ND	III	III	ND	III	ND	III
2	ND	III	III	III	III	III	III	III	ND	III	ND	ND
3	II	II	II	ND	II	ND	II	II	ND	ND	II	ND
4	III	III	ND	III	III	III	ND	III	III	ND	ND	III
8	ND	III	ND	III	III	ND	ND	III	ND	ND	ND	ND
10	ND	III	III	III	ND	ND	ND	III	III	ND	III	ND

ND = No data.

III (n = 5) or II (n = 1). To the best of our knowledge, this is the first report of *T. gondii* DNA in blood and milk samples from donkeys and its genotyping in this host species.

The seroprevalence of *T. gondii* infection in donkeys is frequently high, including seropositivity rates of 45% [17] and 65.6% [18] in Egypt, 43.2% in Brazil [19], 34% in Spain [20], 20.3% [21] and 23.6% [22] in China, from 5 to 8% in Italy [23], and 6.4% in the United States of America [24]. Additionally, milk was found to be positive for *T. gondii* antibodies in 46.3% of pregnant jennies [17]. In our study, *T. gondii* antibodies were found in 25% of serum samples from lactating jennies with *T. gondii* DNA in 13.6% and 6.8% of blood and milk samples from them, respectively. Therefore, a high proportion (27.3%) of lactating seropositive jennies were carrying *T. gondii* DNA in milk. *T. gondii* infection in lactating jennies may result in intermittent excretion of *T. gondii* in milk, as suggested in 3 cases (4, 8 and 10) where the results of n-PCR in blood samples were positive but their results in milk were negative. The stage of *T. gondii* excreted in milk of any animal is unknown, but presumed to be the tachyzoite. We did not make any attempt to determine whether *T. gondii* DNA in lactating jennies was from viable organisms. In a recent report from Iran, 5.7% of raw milk samples from bovine, ovine, caprine, buffalo and camel herds were reported to have viable *T. gondii* as determined by bioassays [12]. Consumption of raw milk has been epidemiologically linked to clinical toxoplasmosis in humans, sometimes with serious consequences [1]. This report adds some hints to this parasite's knowledge, together with results obtained with other approaches [25], even if further studies are needed to determine viability and the stage of *T. gondii* excreted in milk.

Results of the present study and those of others [12] suggest the possibility of transmission of toxoplasmosis to humans following consumption of raw milk from animals other than the goat.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FM designed and performed experiments. RP and CDA participated in designing the study. FM and SN analyzed data and wrote the manuscript. LM carried out PCR detection and genotyping, MM, IA and FS performed fieldwork, SN carried out serological analysis, RP revised results, and JPD critically revised the manuscript. All authors read and approved the final manuscript.

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