A preliminary study on the quality and safety of milk in donkeys positive for *Toxoplasma gondii*.

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Running Head: Quality of milk in donkeys positive for Toxoplasma.

Abstract

Toxoplasmosis is one of the five parasitic diseases considered as a priority for public health action. The consumption of raw milk products represents a possible risk, in particular for certain categories of people. The aim of this study was to evaluate the possible effects of *T. gondii* on milk yield and quality in sero-positive animals with parasitemia. Eighteen healthy lactating Amiata jennies, between 90 to 180 days were included in the study. Four donkeys scored positive for Immunofluorescent Antibody Test (IFAT), and each IFAT positive donkey presented parasitic DNA both in the blood and milk. No significant differences were found between milk yield in PCR-positive donkeys compared to the negative cases, however the former tended to have a greater production. Milk quality in the positive donkeys showed a significantly lower percentage of casein (0.72 vs 0.81%) and ash (0.32 vs 0.37%). Positive cases had a highly significant larger average diameter of globules (2.35 µm) and a fewer globules/ mL (2.39 x 10^8). Somatic cell and bacterial counts were normal and in agreement with the literature. *Toxoplasma gondii* did

not seem to present clinical forms in lactating jennies. Further in vivo studies are needed to further assess the risk of *T. gondii* transmission through donkey milk, together with the impact of different stages of infection on milk quality.

Keywords: food safety, milk quality, donkeys, toxoplasmosis

Implications:

This work provides preliminary information on the infection by *Toxoplasma gondii* in donkeys. Toxoplasmosis is a zoonotic infection and clinical forms of toxoplasmosis in humans have been associated with the consumption of unpasteurized goat milk. Furthermore consumption of raw milk products represents a possible risk, particularly for certain categories of people. Currently, relatively little is known about infection by *Toxoplasma gondii* in donkeys. In addition there has been an increase in donkey milk consumption. For these reasons the effects of *T. gondii* on milk safety, yield and quality in sero-positive animals with parasitemia were investigated.

Introduction

In recent years, there has been an increase in donkey milk consumption for humans and it is also used in cosmetics (Faye and Konuspayeva, 2012). Safety for consumers is important, especially considering that often they buy donkey milk raw, directly from farms.

As is well known, the safety of animal products also depends on the health of the livestock. Currently, there has been little monitoring of parasitic diseases in this species and, in particular, relatively little is known about infection by *Toxoplasma gondii* in donkeys (Mancianti et al., 2014). Toxoplasmosis is a zoonotic infection caused by *Toxoplasma gondii*, an opportunistic protozoon belonging to the phylum Apicomplexa. *T. gondii* infections are prevalent in humans and animals worldwide. Up to one third of the world's

54 population is chronically infected (Dubey, 2010) and toxoplasmosis has been targeted by
55 CDC (Center for Disease Control and Prevention) as one of the five top priority parasitic
56 diseases for public health action. Human infections are primarily asymptomatic, but lymph
57 adenopathy or ocular toxoplasmosis can occur in some patients. *T. gondii* infection in
58 pregnant women can lead to miscarriage, stillbirth or other serious consequences in
59 newborns. In immunocompromised patients, toxoplasmosis can be fatal if not treated and
60 the reactivation of a latent infection can cause life-threatening encephalitis (Montoya and
61 Liesenfeld, 2004).
62 The parasite has three primary modes of transmission: via the ingestion of raw meat
63 products containing terminal oocysts, infection by ingestion of sporulate oocysts, and
64 congenital infection. Further infection can also happen through the ingestion of tachyzoites
65 in milk. Clinical forms of toxoplasmosis in humans have been associated with the
66 consumption of raw goat's milk, although it is considered as a secondary mode of
67 transmission (Camossi *et al*., 2011). As previously mentioned, the consumption of raw milk
68 products represents a possible risk, particularly for certain categories of people. The aim of
69 this study was to evaluate the possible effects of *T. gondii* on donkey milk safety, yield and
70 quality in sero-positive animals with parasitemia.
71
72 **Materials and methods**
73 Eighteen healthy lactating Amiata jennies, all adults (7-10 years), with a homogeneous
74 phase of lactation (between 90 days 180 days) were included in the study. All the jennies
75 were semi-extensively reared on the same farm following farming systems typical of the
76 area of origin, based on natural pasture integrated with polyphite hay ad libitum. Between
77 November and December 2012 milk as well as blood samples were taken.
78 An immunofluorescent antibody test (IFAT) was performed on blood samples, using
79 commercially available 12-well slides (VMRD Inc. Pullman, Washington, USA) as the

antigen, and horse-IgG FITC antibody produced in rabbit (Sigma-Aldrich; PBS dilution 1:32). All serum samples were screened with a threshold dilution of 1:20, and the positive sera were end-titrated using two-fold dilutions. Blood and milk (50 ml) specimens from seropositive jennies were processed for molecular analysis (Mancianti *et al*., 2013). A nested-PCR assay was used to screen blood and milk samples for *T. gondii* DNA, as described by Jones *et al*. (2000).

The animals were machine milked and individual morning milk samples were analyzed for: dry matter, fat and lactose by infrared analysis (Milkoscan, Italian Foss Electric, Padua, Italy); proteins, caseins and ashes (A.O.A.C., 1995); somatic cell count (SCC) (Fossomatic, Italian Foss Electric), and total bacterial count (TBC) (plate count agar; 30°C, 72 h). The diameter (µm) and the number of fat globules per mL of milk in each sample were measured by florescence microscopy following a direct method (Martini *et al*., 2013). All the results were analyzed by ANOVA, where a positive scoring for both IFAT in blood and for PCR in blood and milk samples was the fixed effect. Significant differences were considered at the level $P < 0.05$. The statistical analysis was carried out using JMP (2002) software.

Results and Discussion

The IFAT results showed four positive cases of *T. gondii*, and each IFAT positive donkey presented parasitic DNA both in the blood and milk. Recent studies indicate that the elimination of the parasite in milk depends both on the stage of infection and the immune status of the animals. Physiological decreases in peripartum immunity would seem to lead to the resurgence of *T. gondii* tachyzoites from tissue cysts. Tachyzoites can then circulate again and be excreted in milk (Camossi *et al.*, 2011). As is well known, the data concerning the excretion of parasitic DNA in milk does not indicate the presence of live forms. Tachyzoite stages of T. gondii have also been found in milk of several species,

106 including sheep, goats, camels, buffalos and cows; and infection in humans due to the
107 ingestion of raw goat's milk has been documented (Dehkordi *et al.*, 2013). In addition,
108 tachyzoite penetration through the oral-pharyngeal mucosa has been demonstrated in
109 cats. Cats can become infected when high numbers of these parasitic stages are given
110 orally. Tachyzoites are also rapidly killed outside the host, in fact these stages were shown
111 to survive up to 2 h in pepsin solutions (Dubey, 2010).
112 The gross composition of Amiata donkey milk was in agreement with Polidori *et al*. (2009).
113 Although in our study no significant differences were found between morning milk yield in
114 PCR positive donkeys compared to the negative ones, the former tended to have a greater
115 production (Table 1). In addition, the milk quality in the positive animals showed lower ($P < 0.05$)
116 percentages of casein and ash. Changes in milk quality could be linked to the
117 release of enzymes as a result of an antibody response, as shown in mice with *T. gondii*
118 (Chardès *et al*., 1990). According to Evers (2004), antibody responses promote a release
119 of enzymes. This can alter the composition of milk and the fat globule membrane, resulting
120 in variations in diameter. The fat characteristics found in our study are linked with those
121 reported above (Table 2), in fact, positive animals had a larger average globule diameter
122 ($P < 0.01$) and fewer globules/ mL ($P < 0.01$). Some authors have reported that the
123 composition of the membrane, and thus the physical state of the fat, could be useful for
124 monitoring the health status of the mammary gland (Bendixen *et al.*, 2011). However, at
125 the time of milk sampling, in the positive animals there were no clinically forms of mastitis
126 detected, in agreement with findings described in equidae. In addition, somatic cell and
127 bacterial counts were normal and in agreement with the literature. In fact, according to
128 some studies, donkey milk has a strong inhibitory activity against some bacteria due to the
129 high contents of lysozyme and lactoferrin. It should also be highlighted that potentially
130 pathogenic microorganisms have also been isolated in donkey milk with low somatic cell
131 counts (Pilla *et al*., 2010).

In conclusion, *Toxoplasma gondii* did not seem to present a clinical form in lactating jennies, however changes in milk quality were observed, especially regarding caseins, minerals, and fat globules. The present study did not demonstrate that the T. gondii DNA found in milk was from tachyzoites, anyway donkey milk is a potential source of infection for humans considered at risk. Heat treatment of the milk is therefore important before consumption. In the light of these preliminary results, we believe that in vivo studies are needed to assess more thoroughly both the risk of transmission of *T. gondii* through donkey milk and the impact of the various stages of infection on milk quality.

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190 ***Table 1– Quanti-qualitative and hygienic characteristics of Amiata donkey milk***

191 ***positive and negative for Toxoplasma gondii***

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	Positive donkyes (IFAT+ PCR)(n=4)	Negative donkeys (IFAT) (n=14)	r.m.s.e.	Significance (P)
Morning milk yield (mL)	293	367	121	ns
Milk composition (%)				
Dry matter	9.17	9.30	0.25	ns
Fat	0.24	0.31	0.13	ns
Proteins	1.54	1.57	0.03	ns
Casein	0.72	0.81	0.06	< 0.05
Lactose	7.35	7.31	0.16	ns
Ash	0.32	0.37	0.03	< 0.05
Milk hygienic characteristics (log)				
Somatic cell counts	3.39	3.66	0.43	ns
Total bacterial counts	4.04	3.49	0.66	ns

194 ns=not significant; r.m.s.e.= root mean square error

Table 2. Morphometric characteristics of milk fat globules in positive and negative donkeys for Toxoplasma gondii

	Positive donkyes (IFAT+ PCR) (n=4)	Negative donkeys (IFAT) (n=14)	r.m.s.e	Significance (P)
Average Diameter, µm	2.35	1.56	0.37	< 0.05
Number per mL	$2.39X10^{8}$	$3.71X\ 10^{9}$	$1.83\ X\ 10^{9}$	< 0.05
Size categories fat globules (% of the counted globules)				
Small Globules[1]	64.73	85.70	9.94	< 0.05
Medium globules[2]	26.93	13.37	7.56	< 0.05
Large globules[3]	8.34	0.93	3.87	< 0.05

r.m.s.e.= root mean square error

[1] Small Globules with a diameter <2µm

[2] Medium globules with a diameter between 2 and 5 µm

[3] Large globules with diameter >5µm

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