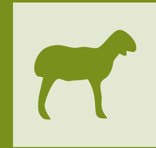
Screening of *Toxoplasma gondii* positive sheep flocks in Perugia Province (Umbria Region, Central Italy) using bulk milk analyses



F. VERONESI[1], F. CHIESA[2], S. ZANET[2], E. BATTISTI[2], V. SEBASTIANI[3], R. BRANCIARI[1], A. VALIANI[4], D. RANUCCI[1]

[1] Department of Veterinary Medicine, University of Perugia, Via San Costanzo 4, 06126 Perugia, Italy
[2] Department of Veterinary Science, University of Turin, Largo Paolo Braccini 2, 10095 Grugliasco (TO), Italy
[3] Free-lance Researcher, Via Giovanni Pagani 34, 67051 Avezzano, L'Aquila, Italy
[4] Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Via Salvemini 1, Perugia, Italy

SUMMARY

Toxoplasma gondii infection in humans is mainly due to consumption of infected raw or undercooked meat and meat products or to accidental ingestion of sporulated oocysts. Nonetheless, human infection could also be caused by the ingestion of unpasteurized milk and milk products. Since ewes' milk is widely used in Italy to produce raw-milk cheese which is locally consumed and world-wide exported, a better understanding of the relevance of the infection rate in sheep herds and the implication as public health threat are needed. Aim of the present paper was to evaluate the prevalence of *T. gondii* in sheep herds located in the Umbria Region (central Italy) through the analysis of ewe bulk milk and by using two different approaches for the screening of infected flocks.

For this tool thirty-six ewe herds located in the Perugia Province were investigated for the presence of *Toxoplasma gondii* antibodies and DNA in bulk milk. Samples were collected at 3 week interval for three times for each flock and analyzed by immunofluorescence antibody test (IFAT) and loop-mediated isothermal amplification (LAMP) methods, for the detection of *T. gondii* antibodies (IgG) and target DNA respectively. Flocks were considered positive to *T. gondii* if at least one bulk milk sample collected tested positive at one of the analytic methods. Twenty-nine flocks were positive with a prevalence of 80.56% but a slight concordance was registered between the two methods considered, in fact the K -value obtained by the agreement analysis between IFAT and LAMP was 0.125. Furthermore, only 30% of the flocks were positive in all three bulk milk samples collected through antibodies, and none by DNA detection. The use of only one method and only one sample, when bulk milk is considered for *T. gondii* screening in sheep flocks, must therefore be strongly discouraged. Further studies are needed to better define control procedure to reduce the prevalence of positive flocks in the investigated areas as well as to better understand the significance for human health of *T. gondii* in ewe milk and products.

KEY WORDS

Toxoplasma gondii, IFAT, LAMP, ewe milk, prevalence.

INTRODUCTION

Foodborne parasites are one of the major burdens for human health in both developing and industrialized countries[1,2]. Despite the decennial knowledge on these parasites, they still represent a challenging task for public health experts[3]. Epidemiological data and appropriate screening surveys are necessary for a reliable risk assessment, for the implementation of control strategies, and for an efficient risk management of all foodborne parasites, including *Toxoplasma gondii*[4]. The importance of *T. gondii* as foodborne parasite is highlighted by a commission of parasitologists that listed it as the second most important hazard among the 24 major foodborne parasites, in terms of their importance for European Countries[3].

Toxoplasma gondii infection in humans is mainly due to consumption of infected meat and meat products[5] or to accidental ingestion of sporulated oocysts[6]. Nonetheless, *T. gondii* infection in humans could also be ascribed to the ingestion of unpasteurized milk and milk products[6]. Excretion of *T. gondii* tachyzoites in milk is reported in goat and infected goat milk is considered as a source of acute infection in humans[7]. Milk derived from other animals is also involved in the horizontal transmission of *T. gondii* infection and may therefore be a source for human infection[8,9]. These data justify the need for a better understanding of the relevance of the phenomenon in sheep herds and the implication as public health threat.

Milk represents a possible matrix for diagnostic screening approaches as both antibodies[10] and DNA of *T. gondii* tachyzoites are present the milk of acutely infected animals[11,12]. Moreover, ewe bulk milk could be readily available for the analytical procedures, as it is commonly sent to laboratories for the assessment of quality and hygienic criteria set by European Legislations (EC Regulation 854/2004)[13].

Previous surveys in the Umbria Region (central Italy) found the presence of *T. gondii* in both pigs' farms and hunted wild boar, highlighting the presence of the parasite in both farmed and wild animals[14,15]. Yet, to date, no data are available for sheep flocks, that are widespread in the Region, with more than 100.000 animals[16], mainly reared outdoor and free range. Furthermore, positive sheep flocks are registered in nearby Regions[17,18]. Ewes' milk is widely used in Italy to produce raw-milk cheeses which are locally consumed and world-wide exported.

Autore per la corrispondenza:
David Ranucci (david.ranucci@unipg.it).

Aim of the paper is to evaluate the prevalence of *T. gondii* in sheep herds located in the Perugia Province (Umbria Region, central Italy) through the analysis of ewe bulk milk and by using two different approaches for the screening of infected flocks.

MATERIALS AND METHODS

The experimental design was built on the basis of the data available for the sheep population and herds in the Umbria Region (central Italy) in particular in the Perugia Province belonging to the aforementioned Region[19]. For the sampling plan, sheep herds were selected among those in the Province of Perugia that were mainly devoted to milk production and with a total number of animals of over 300 units. The 36 farms, possessing these parameters were all tested between June and September 2016. One hundred mL of bulk milk were aseptically collected at farm level from the daily production of each of the 36 flocks. Samples were collected at 3 weeks interval for three times from each flock for a total of 108 samples. Samples were maintained refrigerated and transferred to the laboratory of the Department of Veterinary Medicine of the University of Perugia (Italy), where two aliquots of 50 mL each were prepared. The first aliquot was immediately analyzed for the present of anti-*T. gondii* IgG antibodies by an immunofluorescence antibody test (IFAT). The IFAT was performed using sheep anti-IgG antibodies (Sigma®) conjugated to fluorescein isothiocyanate, a cut off of 1/64, commercial available tachyzoites of the RH strain as antigens (MegaScreen® FLUOTOXOPLASMA g., Diagnostik Megacor) and positive and negative control sera in all the reactions.
The second aliquot was frozen and sent to the Department of Veterinary Medicine of the University of Turin (Italy) for the direct DNA detection by loop-mediated isothermal amplification (LAMP) method according with Trisciuoglio et al.[30]. The LAMP assay used 2 primer pairs targeting the SAG2 gene. The LAMP amplification was optimized in a final volume of 25 L, with 2 L of the extracted DNA, 40 pmol of the FIP and BIP primers, 5 pmol of the B3 and F3 primers, 8 U of *Bst* polymerase in 2.5 L of buffer (20 mM Tris-HCl [pH 8.8], 10 mM KCl, 8 mM $MgSO_4$,10 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 0.8 M betaine, and 1.4 mM deoxynucleotide mix. The LAMP reaction was incubated for 60 min at 65°C and then inactivated at 80°C for 10 min. The resulting amplicons were visualized in 1.5% agar gel using a commercial nucleic acid stain. The LAMP amplicons were also visualized directly in the reaction tube by adding the same diluted fluorescent detection reagent to 2 L of LAMP product. The sensitivity and specificity of the LAMP assays was determined on 10-fold serial dilutions of gDNA of *T. gondii* tachyzoites (10 ng to 1 fg of total gDNA) and tested on heterogeneous DNA samples of *Neospora caninum*, *Babesia* spp., *Theileria* spp., and *Leishmania infantum*. Sterile water was used as negative control, and *T. gondii* gDNA was used as positive control. Positive LAMP products were sequenced at a commercial facility, and the resulting sequences were compared with those available in GenBank, to confirm assay specificity.
Results were registered and the prevalence at farm level was computed with the associated 95% confidence interval (CI). A farm was considered positive if at least one samples among the replicates was found positive with one of the methods adopted. Agreement between IFAT and LAMP on bulk milk was calculated by Kappa statistic (K) and by McNemar's chi-squared test, using 2 x 2 contingency tables where IFAT was considered as the comparative test and LAMP as the alternative test. The statistical significance level was set at $p < 0.05$. The K-values obtained were interpreted according to the following guidelines: < 0.2 = slight agreement, 0.2-0.4 = fair, 0.4-0.6 = moderate, 0.6-0.8 = substantial, > 0.8 = almost perfect[21].

RESULTS

The results of the analyses performed on the bulk milk samples are reported in Table 1. Only 7 out of the 36 flocks were negative using both methods with a total prevalence at herd level of 81% (95% CI = 74-87%); 8 flocks (25%, 95% CI = 15-29%) were positive at both IFAT and LAMP screening.

Table 1 - Percentage of positive flocks to Toxoplasma gondii applying different diagnostic methods.

Method	% of positive flocks	CI 95%
IFAT	55.56	47.39 - 63.72
LAMP	44.44	36.27 - 52.61
Total	80.56	74.05 - 87.06

The disaggregated data about the positive samples registered within the same flock at different sampling time are reported in Table 2. Only in 30% of the flocks, the presence of *T. gondii* antibodies was confirmed in all the three-sampling sequence considered. When samples were tested with LAMP, only in 1 flock the positive result was confirmed even if only in two of the three samples considered, while the remaining flocks were found positive in only one sample. The K -value obtained for the agreement analysis between IFAT and LAMP was 0.125.

DISCUSSIONS

The prevalence of ewe flocks positive to *T. gondii* found in the Perugia Province (Umbria Region) confirm the high prevalence registered in all the other Italian Regions, determined by MAT, IFAT or ELISA, with 77.8% of positive flocks (91 out of 117 flocks) in the Campania Region[11], 87% of the farms (54 out of 62 flocks) in Sicily[22], 87.5% in Lombardy (21 out of 24 flocks)[23] and in Tuscany 97% of the farms (32 out of the 33 flocks) had at least one Toxoplasma-positive animal[18].
The data confirms that the infection is widespread in sheep flocks mainly because of the rearing system adopted, which is partially or totally free-range, with the possibility of infection both from domestic and feral cats as well as from rodent or other wildlife sources[23]. The use of matrixes alternative to sera for the screening of *T. gondii* infection is of utmost importance in all animal species that could transmit the infection to humans through food and is already used in epidemiological surveys[24,25]. Considering the disaggregated data relative to the positive samples within the same flock and the lack of concordance between IFAT and LAMP, the use of bulk milk for the detection of positive flocks appear less effective than sera in sheep, even if strongly recommended by some authors in goat[25]. Milk could be considered a not homogeneous matrix like meat juice[26], and therefore the concordance between positive sera samples and that recorded on bulk milk should be carefully considered for proper epidemiological studies, especially if replicated sampling protocols are

Table 2 - Number of flocks found positive to Toxoplasma gondii during replicated sampling.

Methods	Number of positive flocks	Flocks with 3 positive samples	Flocks with 2 positive samples	Flock with 1 positive sample
IFAT +	20	6 (30% of the positives)	6 (30% of the positives)	8 (40% of the positives)
LAMP +	16	0	1 (6.25% of the positives)	15 (93.75% of the positives)

not adopted. The detection of both antibodies against *T. gondii* or its DNA could be affected by their dilution in the total milk mass. These could be due to a limited number of positive animals within the flocks, but this statement needs further studies to be demonstrated. Nonetheless the prevalence within a heard is known to be limited even in highly infected areas[18]. Eight concordance were registered between the two diagnostic approaches based on antibodies or DNA for determining flocks positive to *T. gondii*. These results are not in agreement with Mancianti et al.[27] that find a perfect concordance between the two methods in goat milk. The analysis of bulk milk from flocks with high number of animals, not all positive to *T. gondii* or not continuously shedding tachyzoites in the milk, could be the cause of this discrepancy.
The adoption of bulk milk as reliable matrix for *T. gondii* screening in sheep flocks needs attention if only one method is adopted along with a sampling protocol without replication.

CONCLUSION

The present survey highlights a high prevalence of *T. gondii* positive ewe flocks in the investigated Italian Province of Perugia, like that found in other Italian Regions. The need for a replicated sampling approach and of the combination of the two methods for determining the actual positivity of sheep flocks, shows that bulk milk is not completely suitable for *T. gondii* screening, despite it being more easily available for testing compared to blood sera. Further studies are needed to better define control procedure to reduce the prevalence of positive flocks in the area as well as to better understand the significance for human health of *T. gondii* in ewe milk and dairy products.

ACKNOWLEDGEMENTS

This research was founded by "Ricerca di Base, 2016" by the Department of Veterinary Medicine of the University of Perugia, Italy.

References

1. Robertson L.J., Sprong H., Ortega Y.R., van der Giessen J.W., Fayer R. (2016). Impacts of globalisation on foodborne parasites. *Trends Parasitol*, 30(1): 37-52.
2. Zolfaghari Emameh R., Purmonen S., Sukura A., Parkkila S. (2018). Surveillance and diagnosis of zoonotic foodborne parasites. *Food Sci Nutrit*, 6(1): 3-17.
3. Bouwknegt M., Devleesschauwer B., Graham H., Robertson L.J., van der Giessen J. W. (2018). Prioritisation of food-borne parasites in Europe, 2016. *Euro Surveil*, 23(9): 1-11.
4. Devleesschauwer B., Dorny P., Faes C., Havelaar A.H., Torgerson P. R., Speybroeck N. (2018). Burden and Risk Assessment of Foodborne Parasites. In Ortega Y.R.: Foodborne Parasites (pp. 341-365). Springer Science and Business Media, Inc., NY, USA.
5. Guo M., Dubey J. P., Hill D., Buchanan R.L., Gamble H.R., Jones J.L., Pradhan A.K. (2015). Prevalence and risk factors for Toxoplasma gondii infection in meat animals and meat products destined for human consumption. *J Food Protect*, 78(2): 457-476.
6. Tenter A.M., Heckeroth A.R., Weiss, L.M. (2000). Toxoplasma gondii: from animals to humans. *Int J Parasitol*, 30(12-13): 1217-1258.
7. Boughattas S. (2017). Toxoplasma infection and milk consumption: Meta-analysis of assumptions and evidences. *Crit Rev Food Sci Nutr*, 57(13): 2924-2933.
8. Spalding S.M., Amendoeira M. R. R., Klein C. H., Ribeiro L. C. (2005). Serological screening and toxoplasmosis exposure factors among pregnant women in South of Brazil. *Rev Soce Bras Med Trop*, 38(2): 173-177.
9. Klauck V., Pazinato R., Radavelli W. M., Custódio E., Bianchi A.E., Camillo G., Cesar A.S., Vogel F.F., Tonin A.A., Ferreira R., Stefani L.M. (2016). Toxoplasma gondii infection in dairy ewes: Vertical transmission and influence on milk production and reproductive performance. *Microb Pathog*, 99: 101-105.
10. Björkman C., Holmdahlù O.J.M., Uggla, A. (1997). An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to Neospora caninum in serum and milk of cattle. *Vet Parasitol*, 68(3): 251-260.
11. Fusco G., Rinaldi L., Guarino A., Proroga Y.T.R., Pesce A., Cringoli G. (2007). Toxoplasma gondii in sheep from the Campania region (Italy). *Vet Parasitol*, 149(3-4): 271-274.
12. Vismarra, A., Barilli, E., Miceli, M., Mangia, C., Bacci, C., Brindani, F., Kramer, L. (2017). Toxoplasma gondii and pre-treatment protocols for polymerase chain reaction analysis of milk samples: a field trial in sheep from Southern Italy. *It J Food Safety*, 6(1): 45-48.
13. EC Regulation 854/2004 of the European Parliament and the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Union, L226.
14. Veronesi F., Ranucci D., Branciari R., Miraglia D., Mammoli R., Piergili Fioretti D. (2011). Seroprevalence and risk factors for Toxoplasma gondii infection on finishing swine reared in the Umbria region, central Italy. *Zoonoses Public Health*, 58(3): 178-184.
15. Ranucci D., Veronesi F., Moretti A., Branciari R., Miraglia D., Manfredi M. T., Piergili Fioretti D. (2013). Seroprevalence of Toxoplasma gondii in wild boars (Sus scrofa) from Central Italy. *Parasite*, 20: 48.
16. IZSUM-ODV Osservatorio Epidemiologico Umbria (2017). Allevamento Ovino e Caprino in Umbria (aggiornamento al 31 dicembre 2017). Available at: http://www.izsum.it/files/Download/112/14868/ ALLEVAMENTO%20OVINO%20E%20CAPRINO%20IN%20UMBRIA_3.pdf
17. De Caprariis D, Gravino E (1981). Diffusione della toxoplasmosi nella specie caprina: indagine sieroepidemiologica su un campione di animali del basso Lazio. *Acta Med Vet*, 27: 261-265.
18. Cenci-Goga B.T., Ciampelli A., Sechi P., Veronesi F., Moretta I., Cambiotti V., Thompson, P.N. (2013). Seroprevalence and risk factors for Toxoplasma gondii in sheep in Grosseto district, Tuscany, Italy. *BMC Veterinary Research*, 9(1): 25.
19. IZSUM-ODV Osservatorio Epidemiologico Umbria (2015). Allevamento Ovino e Caprino in Umbria (aggiornamento al 31 dicembre 2015). Available at: http://www.izsum.it/files/Download/112/14868/ L%E2%80%99ALLEVAMENTO%20OVINO%20E%20CAPRINO%20IN%20UMBRIA.pdf
20. Trisciuoglio A., Zanet S., Marello G., Chiesa F., Nucera D.M., Bergallo M., Gennero M.S., Ferroglio E. (2015). The use of loop-mediated isothermal amplification improves Toxoplasma gondii detection in wildlife. *J Vet Diagn Invest*, 27(6):754-757.
21. Thrusfield M. Veterinary Epidemiology 3rd ed. Oxford, United Kingdom: Blackwell Science Ltd., 2007, pp. 327-328
22. Vesco G., Buffolano W., La Chiusa S., Mancuso G., Caracappa S., Chianca A., Villari S., Currò V., Liga F., Petersen E. (2007). Toxoplasma gondii infections in sheep in Sicily, southern Italy. *Vet Parasitol*, *146*(1-2): 3-8.
23. Gazzonis A. L., Veronesi F., Di Cerbo A. R., Zanzani S. A., Molineri G., Moretta I., Moretti A., Piergili Fioretti D., Invernizzi A, Manfredi M. T. (2015). Toxoplasma gondii in small ruminants in Northern Italy-prevalence and risk factors. *Ann Agric Environ Med*, 22(1): 62-68.
24. Ranucci D., Veronesi F., Branciari R., Miraglia D., Moretta I., Piergili Fioretti, D. (2012). Evaluation of an immunofluorescence antibody assay for the detection of antibodies against Toxoplasma gondii in meat juice samples from finishing pigs. *Foodborne Path Dis*, 9(1): 75-78.
25. Gazzonis, A. L., Zanzani, S. A., Stradiotto, K., Olivieri, E., Villa, L., & Manfredi, M. T. (2018). Toxoplasma gondii antibodies in bulk tank milk samples of caprine dairy herds. *J Parasitol*. In press.
26. Wallander C., Frössling J., Vågsholm I., Burrells A., Lundén A. (2015). "Meat juice" is not a homogeneous serological matrix. *Foodborne Path Dis*, 12(4): 280-288.
27. Mancianti, F., Nardoni, S., D'Ascenzi, C., Pedonese, F., Mugnaini, L., Franco, F., & Papini, R. (2013). Seroprevalence, detection of DNA in blood and milk, and genotyping of Toxoplasma gondii in a goat population in Italy. *BioMed Res Int*, 2013, ID905326: 1-6.