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

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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 is 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 countries2,3. Despite the decennial knowledge on these parasites, they still represent a challenging task for public health experts5. 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 gondii4. 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 Countries4.

Toxoplasma gondii infection in humans is mainly due to consumption of infected meat and meat products5 or to accidental ingestion of sporulated oocysts6. Nonetheless, T. gondii infection in humans could also be ascribed to the ingestion of unpasteurized milk and milk products7. Excretion of T. gondii tachyzoites in milk is reported in goat and infected goat milk is considered as a source of acute infection in humans7. Milk derived from other animals is also involved in the horizontal transmission of T. gondii infection and may therefore be a source for human infection8,9. 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 antibodies8,9 and DNA of T. gondii tachyzoites are present in the milk of acutely infected animals11,12. 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 animals14,15. Yet, to date, no data are available for sheep flocks, that are widespread in the Region, with more than 100,000 animals16, mainly reared outdoor and free range. Furthermore, positive sheep flocks are registered in nearby Regions17,18. Ewes’ milk is widely used in Italy to produce raw-milk cheeses which are locally consumed and world-wide exported.
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 Region26. 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 presence of *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 Ag., Diagnostik M egacor) 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, containing 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**

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>
<thead>
<tr>
<th>Method</th>
<th>% of positive flocks</th>
<th>CI 95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT</td>
<td>55.56</td>
<td>47.39 - 63.72</td>
</tr>
<tr>
<td>LAMP</td>
<td>44.44</td>
<td>36.27 - 52.61</td>
</tr>
<tr>
<td>Total</td>
<td>80.56</td>
<td>74.05 - 87.06</td>
</tr>
</tbody>
</table>

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 Region11, 87% of the farms (54 out of 62 farms) in Sicily22, 87.5% in Lombardy (21 out of 24 flocks)23 and in Tuscany 97% of the farms (32 out of the 33 farms) had at least one Toxoplasma-positive animal18. 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 sources21. 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 surveys22,23. 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 goat21. Milk could be considered a not homogeneous matrix like meat juice26, 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 used.
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 flock; however, this statement needs further studies to be demonstrated. Nonetheless the prevalence within a herd is known to be limited even in highly infected areas.

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 a 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 being it 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

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

<table>
<thead>
<tr>
<th>Methods</th>
<th>Number of positive flocks</th>
<th>Flocks with 3 positive samples</th>
<th>Flocks with 2 positive samples</th>
<th>Flock with 1 positive sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFAT +</td>
<td>20</td>
<td>6 (30% of the positives)</td>
<td>6 (30% of the positives)</td>
<td>8 (40% of the positives)</td>
</tr>
<tr>
<td>LAMP +</td>
<td>16</td>
<td>0</td>
<td>1 (6.25% of the positives)</td>
<td>15 (93.75% of the positives)</td>
</tr>
</tbody>
</table>

References