ISCOM ELISA in milk as screening for Neospora caninum in dairy sheep

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SUMMARY
Introduction - Neospora caninum is recognized as one of the major causes of abortion in cattle and as a causative agent of reproductive disorders in sheep.
Aims - To assess if milk can be used as a matrix for serological investigations of N. caninum in sheep, in the same way as that performed for blood serum, and then to perform an epidemiological survey using bulk tank milk from various sheep farms located in Sardinia (Italy).
Materials and methods - In the first step, individual blood and milk samples of 304 sheep from 5 different farms were tested using a commercial ISCOM ELISA kit. Samples with a Percent Positivity Value (PP%) ≥ 20 were considered positive. In a second step an epidemiological survey was carried out on bulk milk samples collected from 613 sheep farms in Sardinia.
Results and discussion - Antibodies against N. caninum were detected in all of the 5 monitored farms, with positivity of 44.4% and 46.4% in blood and milk samples respectively.
Statistical analysis of the results showed a good correlation between blood and milk samples (r = 0.946; P < 0.001). A TG-ROC plot allowed us to establish that the optimal cut-off for milk analysis was 20.04 PP%.
The present work represents the first sero-epidemiological survey of N. caninum carried out in dairy sheep milk. Sensitivity and specificity obtained with ISCOM ELISA on milk compared with blood serum, indicated that milk from individuals can be a useful tool for large-scale seroepidemiological surveys of N. caninum.
Conclusions - The high level of antibodies against the protozoa found in the bulk milk analyzed suggests that further studies are necessary to assess the best cut-off for analysis of this kind of sample and to understand better the parasitic dynamics in this small ruminant.

KEY WORDS
Dairy sheep, Iscom ELISA, Neospora caninum, Sardinia, Italy.

INTRODUCTION
Neospora caninum is an intracellular coccidian parasite described for the first time in the 1980’s and afterwards recognized as being amongst the major causes of abortion in cattle. Neospora spp. belongs to the Phylum Apicomplexa and its life cycle involves two hosts: a definitive host, generally domestic canids, and an intermediate host, amongst which the most common is cattle. Sheep, goats, equids and wild ruminants, and the dog itself, could also be intermediate hosts, whilst humans are not regarded as intermediate hosts of N. caninum.
Carnivores can be infected through ingestion of tissues of intermediate hosts containing bradyzoites, whilst the intermediate host is infected through ingestion of oocysts, or by transplacental transmission of tachyzoites from the dam to the foetus.
Cows of any age may abort from three months of gestation up to full term, with most abortions occurring at five to six months gestation.
In sheep, N. caninum was first diagnosed in a congenitally infected lamb in England.
Neosporosis in sheep appears to have a cycle similar to that observed in cattle, although the disease is less well studied. Under experimental infections pathological events during pregnancy, similar to those detected in bovine neosporosis, were observed.
The current seroepidemiological situation of neosporosis in sheep is rather heterogeneous, with prevalences ranging from 0.45% to 15% in flocks without reproductive problems. A great increase in seroprevalence, from 23% up to 63%, was observed in farms with abortions/reproductive problems.
Little data for Italy is available, one study reporting a prevalence of 2% in a study that included 1010 domestic sheep that showed no abortion anamnesis.
In Sardinia, the first and only survey on sheep neosporosis reported the presence of DNA of N. caninum, detected by PCR, in 2% of aborted sheep foetuses.
Sardinia is a suitable place for carrying out an epidemiological survey on sheep, as almost 3,228,878 dairy sheep (Mini-
stero della Sanità, 2013) are raised on the island, producing 3.2% of world’s sheep milk (Agenzia Laore Sardegna, 2012). Animals are generally bred with extensive methods, in a very similar way to that of other Mediterranean countries, so the island could be an excellent model for trials and epidemiological studies.

ISCOM ELISA kits® have been successfully used in cattle on blood serum as well in individual and tank bulk milk for the serodiagnosis of neosporosis, obtaining a very good correlation between the two matrices in different part of the world16, 19, including Sardinia20. Considering this, and the complete lack of information on *N. caninum* in dairy sheep, the goals of this survey were to perform a seroepidemiological investigation, in Sardinia, in order to evaluate the correlation between *Neospora* antibodies in serum and sheep milk for diagnostic purposes, to establish the best cut-off for milk analysis and to obtain epidemiological data on sheep neosporosis in Sardinia.

**MATERIALS AND METHODS**

**Serum and milk ISCOM ELISA comparison**

In 2013 a seroepidemiological survey was performed in five different sheep farms in Sardinia, Italy (39° 13' 0'' N, 9° 7' 0'' E) in the provinces of Nuoro (Farms A and C), Sassari (Farm B), Oristano (Farm D) and Cagliari (Farm E) to compare the performances of the ISCOM ELISA kit® (Svanova Biotech AB®, Uppsala, Sweden) in individual milk and the corresponding blood samples from sheep.

Farms were selected according to practitioner veterinarian remote anamnesis, results from which showed that in four of these farms (B, C, D, E) there were no problems of fertility and/or abortions, whilst in farm A there was a history of recurrent abortions from the 2010/2011 season, involving about 20% of adult ewes and almost all the replacement sheep at their first pregnancy.

Three hundred and four (304) individual blood samples from adult and primiparous Sarda breed sheep were sampled from the five farms, and at the same time milk was withdrawn from the two half-breasts of the same animal.

Blood samples were centrifuged at 1,000 ×g for 10 min and serum collected. Milk samples were skimmed by centrifuging them at 3,000 ×g for 3 min at -9°C. Sera and skimmed milk were stored at −20°C±2°C and then analysed using the ISCOM ELISA kit® (Svanova Biotech AB®, Uppsala, Sweden) in individual milk and the corresponding blood samples from sheep.

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**RESULTS**

**Serum and milk ISCOM ELISA comparison**

Seropositivity to *N. caninum* was detected in 44.4% of the animals examined and in all of the farms monitored, with seroprevalence values ranging from 2.7% in Farm B, 2.9% in Farm D, 30.8% in Farm C, 46.2% in Farm E, to 66.9% of the Farm A. The differences in seroprevalence found between the farms were statistically significant ($\chi^2 = 85.04; P < 0.0001$) (Table 1).

For milk samples, our investigation has allowed to the detection of anti *N. caninum* in 46.4% of sheep examined and in all of the farms monitored. However, in comparison to seroprevalences, the test allowed the detection a very similar picture to that obtained through the blood serum, with Optical reading was performed and, for each sample, Percent Positivity Value (PP) was calculated as follows: PP= OD Sample / OD Positive Control X 100. Samples with PP (%) ≥ 20 were considered positive.

The performance of the test on milk was evaluated using two graph receiver-operating characteristic (TG-ROC) analysis16, testing the agreement between milk and serum, and calculating the specificity (SP) and sensitivity (SE) of milk versus serum.

### Epidemiological survey

To assess the epidemiological situation of dairy sheep farms of Sardinia, bulk milk samples from 613 sheep farms from 146 different municipalities of Sardinia were analysed. The farms sampled were distributed as follows on the island: 125 from Sassari Province, 186 from Nuoro, 52 from Oristano and 250 from Cagliari Province.

Bulk tank milk from each farm was sampled and skimmed as described above and then stored at -20°C.

As in the first step of the survey, the *Neospora* ISCOM ELISA kit® (Svanova Biotech AB, Uppsala, Sweden) was then used for the detection of anti *N. caninum* in each sample.

### Data analysis

Data obtained was processed using the software Excel® (Microsoft Corp., Redmond, WA, USA), the *chi-square* tests performed using Epi-Info® (version 7.0, CDC/WHO, Atlanta, GA, USA), whilst the Pearson’s correlation and the TG_ROC analysis were performed using IBM® SPSS® Statistic 20 (IBM Corporation 1989, 2011).

### Table 1 - Animals examined and prevalences of *Neospora caninum* in serum and milk.

<table>
<thead>
<tr>
<th>Farm</th>
<th>Number examined</th>
<th>Serum</th>
<th>Milk</th>
<th>Pearson correlation Bulk milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos Average PP%</td>
<td>Seroprev CI 95%</td>
<td>Pos Average PP%</td>
<td>Prev CI 95%</td>
</tr>
<tr>
<td>A</td>
<td>154</td>
<td>103</td>
<td>62.23%</td>
<td>66.90%</td>
</tr>
<tr>
<td>B</td>
<td>37</td>
<td>1</td>
<td>7.01%</td>
<td>2.70%</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>12</td>
<td>19.46%</td>
<td>30.80%</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>1</td>
<td>13.19%</td>
<td>2.90%</td>
</tr>
<tr>
<td>E</td>
<td>39</td>
<td>18</td>
<td>24.28%</td>
<td>46.20%</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>135</td>
<td>39.51%</td>
<td>44.40%</td>
</tr>
</tbody>
</table>
the Farm A showing a prevalence of 66.9%, whilst Farms B, C, D and E showing prevalences of 24.3%, 46.2%, 14.3% and 15.4% respectively ($\chi^2 = 62.83; P < 0.0001$) (Table 1).

The average of all PP values for blood serum and milk samples are reported in Table 1.

Statistical analysis of the results obtained for blood and milk of samples from individual animals showed a correlation of 0.946 ($P = 0.000$). The specificity of the test performed with skimmed milk, compared to that obtained for blood (our gold standard), was 96.45%.

The TG-ROC plot showed that a cut-off of 20.04 was the value where sensitivity and specificity were 100% (Figure 1). The TG-ROC curve also showed that any cut-off between 18.9 and 20.6 would provide a minimum of 96% sensitivity and 96% specificity for the ELISA kit.

**Epidemiological survey on bulk milk**

*Neospora caninum* antibodies were detected in all the farms monitored in Sardinia through bulk tank milk analysis, even with very different PP values.

The 44.2% of farms show PP values $\geq 20$. Considering that PP% ≥ 20 is the cut-off used for serological diagnosis in individual samples, and that the serum of positive animals would be diluted in the tank, we can say that farms showing PP% ≥ 20 in their bulk tank milk would definitely have *Neospora* seropositive animals in their flocks. The results grouped by class of PP% are detailed in Figure 2.

The five farms investigated in the first step of this study were also examined for bulk tank milk analysis and the results were reported in Table 1.

**DISCUSSION**

The present work represents the first seroepidemiological survey on *N. caninum* performed for dairy sheep milk. The sensitivity and specificity obtained with the ISCOM ELISA on milk, compared with blood serum, indicates that milk from individual animals can be a useful matrix for seroepidemiological surveys of *N. caninum*. The high correlation of serological results between blood and milk, also suggests the validity of this test for whole flock bulk tank milk analysis, as has already performed for cattle in previous studies. Further surveys should be carried out in order to understand how this kind of approach works in other epidemiological situations, such as in non-endemic areas. The use of milk instead of blood serum could allow a first screening of very large territories without an invasive intervention inside sheep farms, which is particularly appreciated by farmers. Also because every kind of therapy or screening that involves contact with...
animals is usually deemed by farmers as a stressor, which usually impacts on production, particularly of milk. The prevalence values obtained in the five Sardinian farms in the first part of this study were far higher than those found in similar epidemiological investigations carried out on animals that showed no reproductive diseases (where seroprevalence is usually around 10%). The data herein seems closer to those found in surveys carried out on farms experiencing reproductive problems, such as 57% reported in New Zealand\(^2\) and 63% in Northern Jordan\(^3\), results for those studies having been obtained through seroepidemiological surveys carried out on blood samples. The results of bulk milk analysis, the first step in this study, are reported in table 1. It is interesting to note how the average PP\% of individual milk samples, undergo a dilution-type compared with bulk milk, showing values two to five times lower than the “Bulk milk PP\%”. The analysis of bulk milk enabled the detection of the farms showing a PP\% ≥ 20 (44.2%) and, therefore, certainly containing more than one positive sheep inside their flocks. Further studies are necessary to establish the best cut-off value for the bulk milk analysis. Whilst in this study a “Bulk milk PP\%” of 34% in farms with reproductive problems (Farm A) in which prevalences of 67% and an “Average PP\%” of 89% were recorded, it would be better to clinically investigate all farms with PP values >20. The serological screening of the farms through the use, not only of individual milk, but also of bulk tank milk, allows the identification farms with the highest seropositivity using a very fast and simple test and then later to extending the diagnosis with more sensitive and specific methods.

Compared to what was observed in cattle in Sardinia, where the Neosporosis is more frequently reported in the provinces where high production performance dairy cattle were raised\(^4\), the higher prevalences in sheep were reported in Nuoro (49%) and Sassari (47%). According to our data, it seems that the diffusion of the parasite is different in the two animals species. In particular, it would seem that the trend of seroprevalence in sheep is more similar to that observed in cattle extensive farms rather than intensive ones. This agrees with the typical sheep breeding in Sardinia, which is carried out with semi-extensive methods, like in other Mediterranean countries. Both kinds of extensive breeding (sheep and cattle) also harbour the definitive and intermediate hosts of this and other important parasitic diseases\(^2,23\).

In addition to these aspects, it must be considered that sheep farming in the Mediterranean area, although in a crisis situation, has in the few last years pushed for increasingly higher levels of production. This is often not supported by proper feeding and management, inevitably creating stress situations and a lower resistance to opportunistic pathogens, such as N. caninum, which have been present on the island for a long time, but confused with other noxae or was not so virulent in the past.

CONCLUSIONS

Based on the evidence of high prevalences herein reported, N. caninum seems to be a widespread parasite in Sardinian sheep farms. Further extended studies should be performed in the future, especially on the farms that have higher PP values, to assess whether these values should be associated with reproductive disorders or not.

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References