Seroprevalence of *Toxoplasma gondii* in domestic sheep in Belgium

D. Verhelst\(^a\), S. De Craeye\(^b\), M. Vanrobaeys\(^c\), G. Czaplicki\(^d\), P. Dornye\(^e\)\(^,f\), E. Cox\(^a\)

\(^a\) Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium
\(^b\) National Reference Laboratory for Toxoplasmosis, Operational Direction Communicable and Infectious Diseases, Scientific Institute of Public Health, Federal Public Service Public Health, Security of the Food chain and Environment, Brussels, Belgium
\(^c\) Animal Health Care Flanders, Torhout, Belgium
\(^d\) Animal Health Care Wallonia, Cine, Belgium
\(^e\) Department of Biomedical Sciences, Institute for Tropical Medicine, Antwerp, Belgium
\(^f\) Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium

**Abstract**

Even though infected sheep are a potential source of *Toxoplasma gondii* infection in humans, information is lacking concerning the seroprevalence of *T. gondii* infection in sheep in Belgium. We examined 3170 serum samples for anti-*Toxoplasma* IgG in sheep by total lysate antigen (TLA) enzyme-linked immunosorbent assay (ELISA). IgG to *T. gondii* was demonstrated in 87.4% of the tested sheep and in 96.2% of the 209 tested flocks. The seroprevalences in Antwerp (65.2%) and Wallonia (68.6%) are statistically lower than in the other regions in Belgium (96.7–97.8%) \((P<0.05)\). The present study is the first report that analyzed the prevalence of *T. gondii* infection in sheep in Belgium and confirms the high prevalence of *Toxoplasma*-specific IgG antibodies in the sheep population.

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1. Introduction

*Toxoplasmosis* is a worldwide zoonosis caused by the obligatory protozoan *Toxoplasma gondii*. Almost all warm-blooded animals and humans can be infected (Yuan et al., 2013). Infections with *T. gondii* in sheep may lead to abortion and causes huge economic losses to the livestock industry (Tenter et al., 2000). In addition consumption of non-frozen and undercooked sheep meat products are a source for human toxoplasmosis (Cook et al., 2000). The prevalence of *T. gondii* infection in sheep has been reported for many countries and ranged from 10% in Slovakia (Kováková, 1993) to 27.8% in the Netherlands (Opsteegh et al., 2010), 65.6% in France (Dumetre et al., 2006) and even 98% in Egypt (Ghoneim et al., 2010). Considering the importance of *T. gondii* in combination with the lack of knowledge about the prevalence in Belgium, the aim of this study was to determine the prevalence of *T. gondii* infection in sheep and sheep flocks in Belgium.

2. Materials and methods

2.1. Sample selection

Blood samples were collected from domestic sheep participating in the Visna-Maedi/CAE screening programme of the Federal Agency for Safety of Food Chain (FASFC). These
samples were from animals older than one year and from flocks from East Flanders, West Flanders, Antwerp, Limburg, Flemish Brabant, and Wallonia. The distribution of samples over sheep flocks of the different provinces and the number of sheep per provinces are presented in Table 1. After testing for Visna-Maedi/CAE the 3170 serum samples were kept at −20 °C until analysis for *T. gondii* antibodies in this study.

The blood samples were marked per flock and were not marked for specific characteristics such as age, breed, gender and farm management. The blood samples were tested for IgG antibodies to *T. gondii*, based on a *T. gondii*-specific antibody enzyme-linked immuno-sorbent assay (ELISA) following a protocol described further. One hundred samples were tested in indirect immunofluorescence assay (IIFA) to confirm the results of the total lysate antigen (TLA) ELISA. The agreement between the results of the TLA ELISA and the IIFA was assessed by calculating positive and negative agreement indices with credibility intervals according to the method described by Graham and Bull (1998) (Table 2). Considering the results of those two tests, *a*, *b*, *c* and *d* are the observed results for each possible combination of ratings by the tests. *a*, being the number of positives with both tests, *b*, the number of samples testing negative in ELISA but positive in IIFA, *c*, the number of samples testing positive in ELISA but negative in IIFA, *d*, the number of negative samples in both tests. The proportion of specific agreement, positive agreement index (pr+) and negative agreement index (pr−) were calculated as follows: pr+ = 2a/(2a + b + c) and pr− = 2d/(2d + b + c). The positive rating is an estimation of the conditional probability, given that one of the test results is positive, the other is also positive. The true prevalences were estimated in a Bayesian analysis based on the model used by Speybroeck et al. (2013). The apparent antibody status of a sheep was linked to the true antibody status in terms of sensitivity (Se) and specificity (Sp) of the diagnostic test. Although we found a 100% agreement between the IIFA and the TLA ELISA, we obtained the Se and Sp estimation of those tests from a previous study (Shaapan et al., 2008). The ELISA had a high sensitivity of 90.1% and the IIFA 80.4%. On the other hand, the specificity of the ELISA (85.9%) was lower than that of the IIFA (91.4%) (Shaapan et al., 2008). According to this study a Se range between 0.80 and 0.90 and a Sp range between 0.85 and 0.90 was used for the TLA ELISA. Credibility intervals (95%) of the true prevalences were calculated using the Bayesian model in Prevalence Package for R version 0.2.0 (Devleesschauwer et al., 2013; Speybroeck et al., 2013).

### 2.2. *T. gondii*-specific antibody enzyme-linked immunosorbent assay (ELISA)

In this ELISA, serum samples were tested for antibodies against total lysate of *T. gondii*. TLA was prepared as described by Scorza et al. (2003). Briefly, tachyzoites of the *T. gondii* RH strain, isolated from the peritoneal cavity of Swiss mice 4 days after intraperitoneal infection, were squeezed twice through a 26-Gauge needle and pelleted by centrifugation at 1000 × g. Next, the pellet was washed twice in PBS, followed by freezing, thawing and sonication on ice for 5 min in an ultrasonic disintegrator (MSE, Leicester, United Kingdom) to solubilize the *T. gondii* tachyzoite antigens. The ELISA was performed as described by Verhelst et al. (2011). Briefly, 96-well Nunc Maxisorp™ plates were coated overnight with TLA at a concentration of 100 μg ml⁻¹ bicarbonate coating buffer (pH 9.4) at 4 °C, blocked with PBS 5% non fat milk powder for 2 h at 37 °C and subsequently with 100-fold dilutions of the serum samples in PBS 0.2% Tween® 20 for 1 h at 37 °C. Thereafter, an anti-sheep IgG horseradish peroxidase conjugated rabbit antiserum (AbD Serotec, Belgium) appropriately diluted in PBS 0.2% Tween 20® was added for 60 min at 37 °C. In between each step plates were washed 5 times with PBS 0.01% Tween20®. As a last step, a 3,3,5,5-tetramethylbenzidine buffer was added for 30 min at room

### Table 1
Seroprevalence of *T. gondii* in sheep and sheep flock in different Flemish provinces and in Wallonia as determined by a TLA-specific antibody ELISA.

<table>
<thead>
<tr>
<th>Province</th>
<th>Flocks</th>
<th>Sheep</th>
<th>True prevalence (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>N tested</td>
<td>N pos</td>
</tr>
<tr>
<td>Flandres</td>
<td>2105</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Antwerp</td>
<td>7127</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>East Flanders</td>
<td>2973</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Flemish Brabant</td>
<td>1783</td>
<td>20</td>
<td>19</td>
</tr>
<tr>
<td>Limburg</td>
<td>5120</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>West Flanders</td>
<td>8145</td>
<td>51</td>
<td>44</td>
</tr>
<tr>
<td>Wallonia</td>
<td>27,253</td>
<td>209</td>
<td>201</td>
</tr>
</tbody>
</table>

### Table 2
Results of the serological tests on sheep sera. Comparison between the TLA-specific antibody ELISA and the IIFA.

<table>
<thead>
<tr>
<th>Activity</th>
<th>IIFA</th>
<th>TLA ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>0 (b)</td>
</tr>
</tbody>
</table>

Positive agreement index: pr+ = 2a/(2a + b + c) = 2 × 63/(2 × 63 + 0 + 0).

Negative agreement index: pr− = 2d/(2d + b + c) = 2 × 37/(2 × 37 + 0 + 0).
temperature, whereafter the OD at 450 nm was determined with an ELISA reader. Positive and negative control serum samples at the same dilution of the serum samples were included on each plate. A corrected optical density (OD) was calculated as the OD of a sample – OD of the negative control samples.

2.3. Indirect immunofluorescence assay (IIFA)

One hundred samples were tested in IIFA to confirm the results of the ELISA. Fifty microliters of the 1/50 diluted serum in PBS was applied for 30 min at 37 °C on a slide coated with formalin-fixed tachyzoites from the RH strain (Toxo-Spot IF, Biomérieux, Marcy-l’Etoile, France). In subsequent steps, the slides were washed with PBS, incubated for 30 min at 37 °C with 30 μl of a 1/25 in PBS-Evans Blue diluted fluorescein isothio-cyanate (FITC) conjugated rabbit anti-sheep IgG (KPL, Maryland, USA), washed again, dried and examined by fluorescence microscopy.

3. Results and discussion

In total, 3170 serum samples from sheep were tested by TLA ELISA for the presence of anti- T. gondii IgG. When comparing the one hundred samples tested in IIFA and TLA ELISA, positive and negative agreement indices with their 95% credibility intervals (between brackets) were used to assess the agreement between the both tests (Table 2). A positive agreement of 1 (0.94–1.00) and a negative agreement of 1 (0.91–1.00) were indicated. The agreement is nearly similar in the negative than the positive direction. Although we found 100% agreement between both tests, the literature mentioned estimation between 90.1% and 97.8% for the sensitivity and between 85.9% and 96.4% for the specificity of the TLA ELISA (Opsteegh et al., 2010; Shaapan et al., 2008).

These 3170 ovine serum samples obtained from DGZ and ARSIA were participating in the Visna-Maedi/CAE screening program of the Federal Agency for Safety of Food Chain (FASFC). As mentioned above, no further information was available about farms, flock size, age, gender or breed. Table 1 gives an overview of the sheep population in Belgium, and shows the results of the tested sheep flocks and the seroprevalence per province. A very high prevalence of 87.4% (CI 82.0–93.4%) was estimated, much higher than previously reported in the Netherlands (27.8%) (Opsteegh et al., 2010) and higher than in other European countries as France with a seroprevalence of 65.6% (Dumetre et al., 2006) or Great-Britain with 74% (Hutchinson et al., 2011). Flanders has 64.33% of the total sheep population and 70.11% of the flocks. Only 8145 flocks are located in Wallonia, the French-speaking southern region of Belgium, which makes 55% of the Belgian territory. The Flemish provinces East and West Flanders are the largest domestic sheep producers. To evaluate whether the seroprevalence in Belgian sheep showed a geographical distribution, the results were grouped per region. Small regional differences were observed and are shown in Table 1. The province of Antwerp showed the lowest seroprevalence (65.2%), followed by the Walloon region (68.6%), East Flanders (96.7%), West Flanders (96.8%) and Limburg (97.3%). Flemish Brabant noted the highest seroprevalence namely 97.8%. It is evident from Table 1 that only 8 of the 209 tested flocks were seronegative. One seronegative flock was located in the Flanders region, more specific in Limburg. The other 7 seronegative flocks were located in Wallonia. The T. gondii seroprevalence in Antwerp and Wallonia were statistically significantly lower than those in the other regions (P < 0.0001) (Table 3).

That almost all flocks in Flanders and 86% of the flocks in Wallonia were seropositive, shows the wide spread of T. gondii in sheep flocks in Belgium.

Interesting in our study is the significantly lower prevalence in Antwerp and Wallonia as compared to the other provinces. Innes et al. (2009a) mentioned that regional differences in the level of soil contamination may lead to variation in the seroprevalence as sheep get T. gondii infections mainly from ingesting oocysts from the environment. It is important in that respect that sporulated oocysts remain viable and infective in the environment for up to 18 months, depending on temperature and relative humidity (Dubey, 1988; Innes et al., 2009b; Owen and Trees, 1999; Plant et al., 1974). Clinical toxoplasmosis in sheep starts mostly by the ingestion of as few as 200 sporulated oocysts (McCoulgan et al., 1988). As sheep are not carnivores, the only two possible infection routes are: (1) the vertical from mother to foetus during pregnancy, that represents a serious risk for congenital disease (Dubey, 2009b), (2) the ingestion of oocysts during grazing. Congenital transmission of T. gondii is reported in several studies (Buxton et al., 2007; Innes et al., 2009a). An infection in the early gestation is mostly fatal due to the absence of a foetal immune response, whereas infection in the late pregnancy, after day 120 of gestation, most of the time results in the birth of normal lambs, which may be infected and immune (Blewett and Watson, 1983). In subsequent pregnancies the effective immune response developed by the infected animals, will protect them against disease (Innes et al., 2009b). Nevertheless, it has been shown that in sheep repeated transplacental transmission of T. gondii can occur (Morley et al., 2005, 2008). The obtained seroprevalence cannot be linked with clinical cases since here are no official records of the prevalence of abortion in sheep in Belgium.

Associations have been made between exposure of sheep to T. gondii and the presence of cats in farms or the circulation of stray cats (Plant et al., 1974). It has been noted that environmental oocyst contamination is concentrated in and around cat defecation sites (Afonso et al., 2008). After a primary infection cats shed continuously oocysts in their faeces for 4–14 days with a peak of tens of millions at 6–8 days (Dubey, 2009a; Dubey and Frenkel, 1972; Skjerve et al., 1998). There are only two reports on the seroprevalence of Toxoplasmosis in Belgian cats, one in the stray cat population of Ghent (Dorny et al., 2002) and one in Belgian pet cats (De Craeye et al., 2008). In the stray cat population of the city of Ghent a very high T. gondii seroprevalence of 70.2% was found (243/346) (Dorny et al., 2002), but this was not the case when testing serum samples of pet cats throughout Belgium (De Craeye et al., 2008). The latter data do not support that cats are the major source of infection for sheep. The lowest seroprevalence in cats was found in West Flanders (12.5%), followed by Antwerp (17%), East
Flanders (21.7%) and Flemish Brabant (21.9%). The highest seroprevalence was noted in the Wallonia and Limburg with 33.2% and 41.7% respectively (De Craeye et al., 2008). Our results showed the highest T. gondii seroprevalence in sheep in Flemish Brabant, Limburg, West and East Flanders. The lower seroprevalence in Antwerp could be explained by the low number of seropositive cats in this region, but on the other hand, there is no correlation between the seroprevalence in cats (33.2%) and sheep (68.6%) in Wallonia. Flock location has been reported as another reason for variation in seroprevalence in sheep (Caballero-Ortega et al., 2008; Halos et al., 2010; Skjerve et al., 1998). However, due to privacy regulations, the exact location of the flocks was not released so that we could not analyze this parameter. We assume that the difference is not due to housing conditions since Belgian sheep are either kept totally outdoor or indoor with outdoor access. The differences we found among the provinces, most likely have to be seen as a result from differences in environmental contamination, but the etiological factors were not identified.

In conclusion, this is the first report of T. gondii seroprevalence in sheep in Belgium. The high prevalence in Belgium demonstrate that more attention has to be given to sheep meat as a potential source of toxoplasmosis for humans.

Acknowledgements

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Table 3

<table>
<thead>
<tr>
<th>P-value</th>
<th>Antwerp</th>
<th>East Flanders</th>
<th>Flemish Brabant</th>
<th>Limburg</th>
<th>West Flanders</th>
<th>Wallonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antwerp</td>
<td>–</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.635</td>
<td>0.826</td>
<td>0.933</td>
</tr>
<tr>
<td>East Flanders</td>
<td>–</td>
<td>–</td>
<td>0.635</td>
<td>–</td>
<td>0.978</td>
<td>-0.0001</td>
</tr>
<tr>
<td>Flemish Brabant</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.978</td>
<td>0.978</td>
<td>0.0001</td>
</tr>
<tr>
<td>Limburg</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.86</td>
<td>0.0001</td>
</tr>
<tr>
<td>West Flanders</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.0001</td>
</tr>
<tr>
<td>Wallonia</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
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References


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