The relationship between presence of antibodies and direct detection of *Toxoplasma gondii* in slaughtered calves and cattle in four European countries

M. Opsteegh[a*], F. Spano[b], D. Aubert[c], A. Balea[d], A. Burrells[e], S. Cherchi[b], J.B.W.J. Cornelissen[f], C. Dam-Deisz[a], J. Guitain[g], A. Györke[d], E.A. Innes[e], F. Katzer[e], G. Limon[g], A. Possenti[b], E. Pozio[b], G. Schares[h], I. Villena[c], H.J. Wisselink[f], J.W.B. van der Giessen[a]

[a]National Institute for Public Health and the Environment, Centre for Infectious Disease Control, P.O. Box 1, 3720 BA Bilthoven, The Netherlands (marieke.opsteegh@rivm.nl (*corresponding author), cecile.dam@rivm.nl, joke.van.der.giessen@rivm.nl)

[b]Department of Infectious Diseases, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy (furio.spano@iss.it, simona.cherchi@iss.it, alessia.possenti@iss.it, edoardo.pozio@iss.it)

[c]National Reference Centre for Toxoplasmosis, Laboratory of Parasitology, EA 7510, University of Reims Champagne-Ardenne and CHU Maison Blanche, Reims, France (ivillena@chu-reims.fr, daubert@chu-reims.fr)

[d]Parasitology and Parasitic Diseases Department, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, 3-5 Mănăştur Street, 400372, Romania (adriana.gyorke@usamvcluj.ro, anamariabalea@yahoo.com)

[e]Moredun Research Institute, Pentlands Science Park, Penicuik, EH26 0PZ, United Kingdom (Frank.Katzer@moredun.ac.uk; Alison.Burrells@moredun.ac.uk; Lee.Innes@moredun.ac.uk)

[f]Wageningen Bioveterinary Research, P.O. Box 65, 8200 AB Lelystad, The Netherlands (jan.cornelissen@wur.nl, henk.wisselink@wur.nl)

[g]The Royal Veterinary College, Veterinary Epidemiology, Economics and Public Health Group, Hawkshead Lane, North Mymms, AL9 7TA, Hatfield, United Kingdom (glimon@rvc.ac.uk, JGuitian@rvc.ac.uk)

[h]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Südufer 10, 17493 Greifswald - Insel Riems, Germany (Gereon.Schares@fli.de)

Note: Supplementary data associated with this article

Abstract

In cattle, antibodies to *Toxoplasma gondii* infection are frequently detected, but evidence for the presence of *T. gondii* tissue cysts in cattle is limited. To study the concordance between the presence of anti-*T. gondii* IgG and viable tissue cysts of *T. gondii* in cattle, serum, liver and diaphragm samples of 167 veal calves and 235 adult cattle were collected in Italy, the Netherlands, Romania and the United Kingdom. Serum samples were tested for anti-*T. gondii* IgG by the modified agglutination test (MAT) and p30 immunoblot. Samples from liver were analyzed by mouse bioassay and PCR after trypsin digestion. In addition, all diaphragms of cattle that had tested *T. gondii* positive (either in bioassay, by PCR on trypsin-digested liver or serologically by MAT) and a selection of diaphragms from cattle that had tested negative were analyzed by magnetic capture qPCR (MC-PCR). Overall, 13 animals were considered positive by a direct detection method: 7 out of 151 (4.6%) by MC-PCR and 6 out of 385 (1.6%) by bioassay, indicating the presence of viable parasites. As cattle that tested positive in bioassay tested negative in MC-PCR and vice-versa, these results demonstrate a lack of concordance between the presence of viable parasites in liver and the detection of *T. gondii* DNA in diaphragm. In addition, the probability to detect *T. gondii* parasites or DNA in seropositive and seronegative cattle was comparable, demonstrating that serological testing by MAT or p30 immunoblot does not provide information about the presence of *T. gondii* parasites or DNA in cattle and therefore is not a reliable indicator of the risk for consumers.

Key words: *Toxoplasma gondii*, cattle, serology, mouse bioassay, PCR, detection

1. Introduction

Toxoplasma gondii is a protozoan parasite that can cause abortion or the birth of an affected child (e.g. with hydrocephalus or intracranial calcifications) when a primary infected pregnant woman transmits the parasite to the fetus. In addition, congenitally infected individuals as well as those acquiring infection later in life are at risk of developing chorioretinitis. Moreover, *T. gondii* infection can cause life-threatening disease in severely immune-compromised patients. Overall, *T. gondii* was estimated to contribute 17.6% to the total burden of foodborne disease in Europe in 2010 (Havelaar et al., 2015) and was ranked the fourth (global) and second (Europe) most important foodborne parasite by experts (FAO and WHO, 2014; Bouwknegt et al., 2018). The infection can be acquired through ingestion of tissue cysts in raw or undercooked meat, and through ingestion of oocysts, for example via contact with soil, cat feces or consumption of contaminated vegetables, water or shellfish. Worldwide food was considered responsible for approximately 50% of *T. gondii* infections (Hald et al., 2016) and consumption of meat is an important risk factor for infection (Cook et al., 2000; Belluco et al., 2017).

Despite its importance as a foodborne pathogen, *T. gondii* is not covered by meat inspection. Tissue cysts are of microscopic scale (Dubey et al., 1998), and meat inspection is currently based on palpation, visual examination of several tissues and digestion of muscle tissues from animals susceptible for *Trichinella* spp.. The European Food Safety Authority (EFSA) has published scientific opinions on the feasibility of better controlling foodborne pathogens such as *T. gondii* by modernization of meat inspection, for example, by using herd information to classify batches into risk groups or by implementation of serological screening (e.g. (EFSA, 2011)). However, in case of *T. gondii* in cattle, two important gaps in knowledge exist.

77 Firstly, prioritization of *T. gondii* for bovine meat inspection remained undetermined (EFSA
78 BIOHAZ Panel, 2013). The role of beef as a source of human infection with *T. gondii* is
79 debated, as serological studies demonstrate that antibodies to *T. gondii* are prevalent in cattle
80 (Tenter et al., 2000), but isolation of *T. gondii* by bioassay from naturally infected cattle is very
81 rare (Dubey, 2010). Even recovery from experimentally infected cattle is inconsistent (Dubey
82 and Thulliez, 1993; Esteban-Redondo et al., 1999; Burrells et al., 2018). On the other hand,
83 consumption of beef has been identified as a risk factor (Baril et al., 1999; Cook et al., 2000;
84 Jones et al., 2009; Belluco et al., 2017; Said et al., 2017) and has been linked to outbreaks
85 (Smith, 1993). In addition, based on quantitative risk assessment, beef was predicted to be the
86 most important source of meatborne infections in the Netherlands and Italy (Opsteegh et al.,
87 2011a; Belluco et al., 2018).
88
89 Secondly, the value of serological screening for *T. gondii* in cattle is unclear. If beef is an
90 important source of *T. gondii* infections, serological assays would be preferred for screening as
91 they allow high-throughput testing at low costs. However, serological screening is of limited
92 use for consumer protection if there is no concordance between detection of antibodies and the
93 presence of viable tissue cysts. In cattle, such a lack of concordance between the presence of
94 antibodies and detection of tissue cysts has been reported (Opsteegh et al., 2011b; Burrells et
95 al., 2018).
96
97 The aims of this study were to determine if *T. gondii* can be detected in slaughtered cattle from
98 different European countries using direct detection methods, and to estimate the concordance
99 between the detection of antibodies using MAT and the detection of *T. gondii* tissue cysts by
100 direct methods. This study provides insight into the role of beef as a risk of *T. gondii* infection

for consumers and the usefulness of serological screening in cattle for estimations of prevalence or to identify herds or animals, which may pose a high risk of *T. gondii* infection for consumers.

2. Material and Methods

2.1. Sample collection

Since a low prevalence of tissue cysts was expected in cattle (Dubey et al., 2005), the sampling plan was designed to maximize the number of cattle that could be sampled, and not to take into account representativeness for cattle slaughtering in Europe. Muscle, liver and blood samples were collected from 100 slaughter cattle for each of the four countries participating in the project, namely Italy, the Netherlands, Romania and the United Kingdom. Because one study conducted in cattle had reported a higher prevalence of *T. gondii* DNA in calves compared to older animals (Berger-Schoch et al., 2011) and it had been suggested that cattle do not readily acquire persistent *T. gondii* infection (Munday, 1978; Munday and Corbould, 1979), we aimed to sample equal numbers of calves and older cattle. Calves were defined as cattle up to 12 months old intended for slaughter as calves (Dec. 94/433/EC). Sampling equal numbers of calves and adult cattle was feasible in Italy, the Netherlands, and Romania, but in the UK slaughtering of calves is very uncommon (3.3% of all slaughtered cattle in 2013, http://beefandlamb.ahdb.org.uk/markets/industry-reports/uk-statistics/) and calves are slaughtered at very young age (≤2 months). Therefore, the sampling target for the UK was set at 16 calves and 84 adult cattle.

To select the most appropriate tissues, a literature review was performed on the anatomical distribution of tissue cysts in cattle (Opsteegh et al., 2016). In this review, no clear predilection sites were identified for *T. gondii* in cattle, but the small intestine and the liver scored highest and skirt steak, lymph nodes, thigh muscle and top round steak scored well based on a limited

number of studies. Liver is easier to collect and more amenable to trypsin digestion for bioassay in mice as compared to small intestines. Therefore, liver was sampled as a predilection site. Diaphragm was sampled as a representative of edible tissue, since it scored similar to the combined muscle and meat category (Opsteegh et al., 2016).

In each country, sample collection was performed at slaughter. At the slaughter line, the investigator would select an animal for sampling when ready with labelling and storage of the previous set of samples, taking into account the limit to one animal per farm and skipping carcasses that were likely to be condemned. Depending on the country, between two and eight slaughterhouses were visited from 2013 to 2014. Cattle were coded with a unique ID and it was ensured that matching samples were labelled correctly. A minimum of 4 ml of blood was collected in a 9 ml serum tube at bleeding or from the heart during evisceration. A minimum of 200 g of the muscular part of the diaphragm and 400 g of liver was collected in separate seal bags. Age, sex and type (dairy, beef or crossbreed) of the animal were noted. Samples were kept and transported on ice or in the refrigerator as much as possible. The liver was processed for mouse bioassay the day after sample collection. Diaphragm samples were stored at -20 °C and, if selected, sent to the National Institute for Public Health and the Environment (RIVM, the Netherlands) for MC-PCR testing.

2.2. Serology

2.2.1. Modified agglutination test

All cattle sera were sent to ANSES – USC EpiToxo in Reims (France) and tested by a modified agglutination test (MAT) as previously described (Dubey and Desmonts, 1987). Sera were diluted two-fold starting at a 1:6 dilution and screened until a dilution of 1:12,800 to detect anti-*T. gondii* antibodies. Both a low cut-off value of ≥1:6 and a more conservative cut-off value of ≥1:100 (suggested by Dubey, 2010) were used to classify samples as positive or negative.

2.2.2. Immunoblot using p30 antigen

Additionally, cattle sera were sent to Friedrich-Loeffler-Institut (Germany) for immunoblotting using affinity purified p30 antigen. To prepare antigen the RH strain of *T. gondii* (Sabin, 1941) was maintained in MARC145 cell cultures and purified as previously described (Pardini et al., 2012; Schares et al., 2017). Cell culture derived tachyzoites were frozen as a pellet at –80 °C until used for antigen purification. The *T. gondii* surface antigen p30 (TgSAG1, SRS29B) was purified by affinity-chromatography using the monoclonal antibody P30/3 (ISL, UK) essentially as described (Hosseininejad et al., 2009; Maksimov et al., 2011; Schares et al., 2017). The immunoblot was performed as previously described (Pardini et al., 2012). Purified p30 (0.5 µg) were incubated in non-reducing sample buffer (2 %[w/v] sodium dodecyl sulfate (SDS), 10 %[v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 min (94°C), separated in 12%[w/v] SDS polyacrylamide minigels of 60 x 70 x 1 mm size and transferred to PVDF membranes (Immobilon-P, Millipore). After the transfer, membranes were blocked using PBS-TG (PBS with 0.05 % (v/v) Tween 20 (Sigma) and 2% (v/v) liquid fish gelatine (Serva, Germany)) and cut into ~50 stripes and examined as described below. Bovine sera were diluted 1:100 in PBS-TG. Peroxidase conjugated anti-bovine IgG (H + L) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used diluted 1:500 in PBS-TG. As positive control we used an ovine pool serum (i.e. a heterologous serum) collected from a naturally exposed sheep (Tzanidakis et al., 2012). The negative control was represented by the serum of a calf born at Friedrich-Loeffler-Institut which tested serologically negative for *T. gondii* by immunofluorescence test (titer <1: 50).

2.3. Mouse bioassay of liver

Mouse bioassay of the liver was performed in the country of sample collection (one laboratory per country). Trypsin digestion of liver and inoculation in two mice per sample was performed as previously described (Villena et al., 2012). Briefly, two-hundred grams of each

liver sample was grounded and mixed with 300 ml 0.25% trypsin (Sigma Aldrich, Trypsin from porcine pancreas T4674) solution in 0.9% NaCl, and supplemented with antibiotics (Penicillin/Streptomycin 60,000 IU/60 mg and Amoxicillin 100 mg). The mixture was incubated for 1.5 hours at 37.0ºC and then filtered through double layered gauze, followed by centrifugation at 1800g for 10 minutes. The supernatant was removed, and the pellet was washed in 0.9% NaCl. Afterwards, 1 ml of the pellet was kept and stored at -20°C for PCR and the remaining pellet was mixed with 1 ml antibiotics solutions (penicillin/streptomycin/amoxicillin and ciprofloxacin/cefotaxime/ vancomycin) and made up to 5 ml total volume with 0.9% NaCl. Per sample, two mice (Swiss Webster of minimum 6 weeks old) per sample were inoculated intraperitoneally with 1 ml of this suspension. Mice were monitored twice daily and given a health score based on coat condition and demeanor. Mice were euthanized based predetermined humane endpoints. Ethical approval for the mice experiments was obtained in the respective countries (details provided in section 2.7). The development of antibodies against *T. gondii* in mice was determined by serology on day 28 in Italy (to prepare for possible strain isolation at day 42) and at post mortem (day 42) in the Netherlands, Romania and the United Kingdom. Mouse sera from the Netherlands and the United Kingdom were tested by ID.Vet ELISA (ID Screen® toxoplasmosis indirect multi-species) and, in case of doubtful results, sent to Reims for confirmation by MAT at 1:25 dilution; all mouse sera from Romania were sent to Reims for MAT without any prior testing; sera from Italy were tested by Toxoscreen DA (BioMerieux). For PCR-based detection in mice, at least half of the mouse brain was homogenized in PBS (75 µl PBS per 25 mg of tissue) and 100 µl of this homogenate was used for DNA isolation as described in the manufacturer's protocol for human or animal tissue and cultured cells (NucleoSpin Tissue, Macherey-Nagel, Germany). The presence of *T. gondii* DNA was investigated by PCR, either qPCR targeting a 529 bp Repeated Element (RE) (Wells et al., 2015) or, in Romania, by

conventional PCR on the 529 bp RE followed by gel electrophoresis (Homan et al., 2000). To consider a mouse brain as qPCR positive, all negative and blank controls in the same PCR run had to be negative, and the Cq-value for the sample had to be <40 with the shape of the amplification curve similar to those of the positive controls. If so, samples with a Cq-value <35 were considered positive, samples with a Cq-value between 35 and 40 were additionally confirmed by identification of the correct band size (162 bp) by gel electrophoresis. Conventional PCR was considered positive when an amplicon of the expected size (529 bp) was identified by gel electrophoresis. A mouse bioassay was considered positive if at least one of the two mice was positive by serology or PCR.

2.4. PCR on liver digest

DNA was isolated from liver digests using the Nucleospin kit (Machery-Nagel) as follows: 200 µl of digest, 1440 µl of T1 buffer and 200 µl of proteinase K (provided with the kit) were mixed and incubated at 56 °C for 1-3 hours. After incubation, 230 µl of the mixture was used for further processing to prevent overloading of the column. The manufacturer's protocol for human or animal tissue or cultured cells was followed as described from step 3 onward (adding 200 µl of B3). Samples were subsequently tested by 529 bp RE qPCR (Wells et al., 2015) or by conventional PCR on the 529 bp RE (Homan et al., 2000). PCR screening of liver digests was performed in the country of sample collection. PCR was considered positive using the criteria described for PCR on mouse brains.

2.5. MC-PCR on diaphragm

MC-PCR was performed on 100 g of the diaphragm from a selection of cattle. The diaphragms from 100 cattle (25 per country) negative by bioassay and PCR on liver digest, but irrespective of their MAT result, were tested by MC-PCR. Next, the diaphragms from all cattle positive by either the mouse-bioassay or the PCR on the liver digest, and the remaining MAT positive

cattle, were additionally tested. All diaphragms were sent to RIVM and stored frozen until tested by MC-PCR as previously described (Opsteegh et al., 2010a). MC-PCR was considered positive using the criteria described for PCR on mouse brains.

2.6. Data analysis

Variation in the proportion of seropositive animals by age category and country was evaluated using multivariable logistic regression analysis using forward selection based on likelihood-ratio test (IBM SPSS Statistics 24). Direct detection rates in seronegatives and seropositives were compared using Fisher's exact test (IBM SPSS Statistics 24). The concordance between the presence of antibodies as determined by MAT and the presence of parasites in bovine tissues was evaluated based on kappa-statistics with 95% confidence interval (winepi.net). For this comparison, mouse bioassay, PCR on liver digest and MC-PCR on diaphragm were considered separately.

2.7. Ethical approval

Animals were housed and maintained according to the European Directive 63/2010 at the Animal Care Unit of the Istituto Superiore di Sanità (ISS) in Italy; at the animal facilities of Wageningen Bioveterinary Research in Lelystad, the Netherlands; at the Laboratory Animals Unit of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania; and at the animal facilities of the Moredun Research Institute, in the United Kingdom.

The *in vivo* protocol was approved by the Italian Ministry of Health (artt. 8 and 9, D.L.vo 116/92, 5th December 2013); by the Animal Ethics Committee of the Animal Sciences Group (Lelystad, the Netherlands) (2014090.d, 24th November 2014); by the Animal Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania (30/2015 USAMV CN); and by the Moredun Experiments and Ethical Review Committee, United Kingdom (E51/14, 30th September 2014).

3. Results

3.1. Features of the samples

A total of 402 slaughtered cattle have been sampled, as two additional cattle as originally planned had been sampled in the UK. The complete dataset is available as a supplement (Table S1). The sex and age distribution of the cattle is presented by country in Table 1. As expected, slaughtered calves were mainly male, whereas slaughtered adult cattle were mainly female. Calves slaughtered in the UK were younger than those from the other countries. In Romania, with the exception of 10 calves, all animals came from backyard farming.

3.2. Detection of anti-*T. gondii* IgG

Antibodies titers ≥1:6 were detected by MAT in 14.9% (95% C.I.: 11.8-18.7%) of cattle. Titers were low (Figure 1), and the highest titer was 1:200 for a 25 month old cow from the UK. Concordance between MAT and p30 immunoblot was low (kappa 0.16, 95% CI 0.10-0.23) (Table 2). Based on MAT with a cut-off of ≥1:6, the seroprevalence was significantly higher in older cattle and varied between countries (Table 3), but seroprevalences are lower and without statistically significant differences when a cut-off value of 1:100 for MAT or immunoblot results are considered.

3.3. Direct detection of *T. gondii*

The presence of *T. gondii* was demonstrated in the tissues of 13 out of 401 cattle (Table 4) (for one adult cattle from the UK no tissue samples were tested). Positive results were always limited to one direct detection method per cattle, i.e. there was no overlap in positive results from mouse bioassay, PCR on liver digest and MC-PCR on diaphragm. As the number of animals positive by a direct detection method was low, statistical evaluation by age, sex or type was not performed.

3.3.1. Mouse bioassay

Six out of 385 (1.6%) cattle tested positive by mouse bioassay (Table 5). Mice were tested serologically and by PCR on the brain, and if either of these methods gave a positive result in one of the two mice inoculated with the same liver digest, the mouse bioassay was scored positive. For one adult cow from the UK no liver sample was collected, and for sixteen calves (<2 months) from the UK, no analysis could be made by mouse bioassay. For 22 cattle the mouse bioassay results were incomplete (e.g. a serological result would be missing when a mouse had to be excluded prior to the end of the bioassay). These 22 cattle were considered mouse bioassay negative based on the available results. With the exception of one cattle liver from Italy, in which both mice of the bioassay tested positive by qPCR on the brain, positive mouse bioassays were based on a positive PCR result for one mouse with Cq-values ranging from 34 to 39. All positive and negative controls in DNA isolation and PCR gave appropriate results.

3.3.2. PCR on liver digest

None of the liver digests tested positive by PCR (Table 5). Positive and negative controls in DNA isolation and PCR gave appropriate results.

3.3.3. MC-PCR on diaphragm

MC-PCR targeting the 529 bp RE of *T. gondii* was performed on diaphragms from 151 cattle. Twenty-five cattle per country were tested irrespective of the results by MAT. In addition, diaphragms from all cattle positive by mouse bioassay, liver digest PCR or MAT, were tested, with the exception of one Romanian cattle with a MAT titer of 1:6, which was omitted by mistake. Seven cattle out of 151 (4.6%), gave a positive MC-PCR result (Table 5) with Cq-values ranging from 35 to 40. These cattle tested negative by mouse bioassay and by PCR on

293 the liver digest, two of them showed positive MAT results of 1:100 and 1:6. Positive and
294 negative controls in DNA isolation and PCR gave appropriate results.

295 **3.4. Concordance between the presence of anti-*T. gondii* IgG and detection of *T. gondii***
296 **in tissues**

297 The probability of direct detection of *T. gondii* in seropositive cattle (MAT ≥1:6) was low (3.3%
298 by mouse bioassay and 5.1% by MC-PCR) and not significantly different from the detection
299 probability in seronegative cattle (1.2% by mouse bioassay and 4.3% by MC-PCR) (Fisher's
300 Exact test, *p*-value = 0.237 and *p*-value =1.000) (Table 5). There is a lack of concordance
301 between presence of anti-*T. gondii* IgG detected by MAT (≥1:6) and detection of *T. gondii*
302 using direct methods: kappa-value 0.03 (95% CI <0-0.21) between MAT and mouse bioassay;
303 kappa-value 0.01 (95% CI <0-0.13) between MAT and MC-PCR. All PCRs on liver digests
304 tested negative (kappa-value = 0.0 between MAT and PCR on liver digest). Considering a cut-
305 off value of 1:100 for MAT or the immunoblot results, the probability of direct detection in
306 seropositives or the concordance between indirect and direct detection did not improve (Table
307 6).

308 **4. Discussion**

309 In this study, tissue and blood samples have been collected from cattle slaughtered in four
310 European countries to study the presence of viable *T. gondii* and its concordance with the
311 presence of anti-*T. gondii* IgG in serum. Ideally, complete carcasses would have been tested
312 using the most sensitive direct detection method, but this is not feasible. Here, three direct
313 detection methods and two types of tissue (liver and diaphragm) were used and 13 of 401 cattle
314 examined were considered positive by a direct detection method. This low number of positive
315 cattle is in correspondence with published data (Dubey et al., 2005; Santos et al., 2010;

316 Opsteegh et al., 2011b; Hosein et al., 2016). In calves, we did not find a high prevalence of
317 directly detected *T. gondii* as reported in Switzerland (Berger-Schoch et al., 2011).

318 Six out of 385 cattle were mouse bioassay positive (1.6%), indicating the presence of viable *T.*
319 *gondii* parasites. As trypsin digestion was used, it is possible that also tachyzoites in addition
320 to tissue cysts have been detected by mouse bioassay (Dubey, 1998). The prevalence of 1.6%
321 is comparable to the MC-PCR based prevalence of 2.0% in cattle in the Netherlands (Opsteegh
322 et al., 2011b) and 1.6% in cattle in the UK (Hosein et al., 2016), but unexpected considering
323 the failure to detect viable *T. gondii* in 2,094 retail beef samples from the USA (Dubey et al.,
324 2005). These six positive mouse bioassay results concerned seven mice, which were positive
325 by PCR using brain samples, but negative by MAT. No clinical signs of toxoplasmosis were
326 observed in any mice, and only one liver digest resulted in positive PCR results for both mice.
327 A lack of agreement between clinical, serological and PCR results for bioassay mice has
328 previously been observed when samples from experimentally inoculated calves where tested
329 using the same bioassay protocol (Burrells et al., 2018). In addition, a lack of concordance
330 between MAT and qPCR on brain has recently been shown also for wild mice (*Mus musculus*
331 *domesticus)* (Galal et al., 2018). Overall, the mouse bioassay results strongly suggest that even
332 when present, the parasite concentration was low in bovine livers: low inoculation doses
333 resulted in low grade infections in mice with low parasite loads in mouse brain homogenates
334 (Cq-values between 34 and 39) and failure to elicit a detectable IgG response in mice.

335 Seven out of 151 cattle (4.6%) tested positive by MC-PCR on the diaphragm. Cq-values were
336 high (between 35 and 40 cycles) and not always repeatable, consistent with a low concentration
337 of *T. gondii* DNA in the diaphragm. For these samples, only the presence of parasitic DNA is
338 demonstrated; therefore, these results do not necessarily provide an indication of risk for
339 consumers.

For all 13 direct detection positive cattle, this conclusion is based on a positive result by qPCR: by MC-PCR on the diaphragm for seven cattle and by PCR on mouse brain for six cattle that were positive in mouse bioassay. We are confident that the positive PCR reactions reflect the presence of *T. gondii* DNA in those samples for several reasons. Firstly, we employed as qPCR target the 529bp repeat element which is specific to *T. gondii* (Homan et al., 2000), and was used to develop a sensitive and specific qPCR (detection limit of 20 fg per PCR reaction) by Reischl et al. (2003), slightly modified by Opsteegh et al. (2010b). Secondly, during the appropriate blank controls included during DNA isolations and PCR runs to rule out potential contamination always confirmed negative. Non-specific amplification was excluded by considering only samples with an amplification curve similar to the positive controls and a Cq-value below 40. Additionally, for samples scoring positive between cycle 35 and 40, the correct amplicon size was confirmed by gel electrophoresis. Sequence confirmation was not applicable due to the low concentration and short size (162 bp) of the amplicons. The low concentration of *T. gondii* DNA also precluded the possibility to get further information on the presence and type of *T. gondii* via genotyping considering the low sensitivity of PCRs based on single copy genes.

Despite the low number of cattle positive by direct detection, the seroprevalence based on MAT with a cut-off value of 1:6 was sufficient to demonstrate a lack of concordance between the presence of IgG against *T. gondii* and direct detection of the parasite using either mouse bioassay, qPCR on liver digest or MC-PCR. The probability to detect *T. gondii* by a direct method was comparable in seropositive versus seronegative cattle, demonstrating that serological testing by MAT should not be used as a proxy for presence of *T. gondii* in cattle.

The concordance between direct and indirect detection can be influenced by the choice of detection methods, as recently shown in chickens (Schares et al., 2018). In the current study, the detection of antibodies in cattle was performed by MAT, a species-independent serological

365 test that is commonly used for bovine sera. The test has been shown to be a proper serological
366 tool in the follow-up of experimentally infected cattle (Dubey et al., 1985), however an in depth
367 evaluation for use with cattle sera in the field is lacking. Cattle sera have additionally been
368 tested for antibodies against p30 (Tg-SAG1) by immunoblot, providing the possibility to
369 discriminate more easily between specific and non-specific reactions. These results did not
370 agree well with MAT or overall direct detection. In addition, Romanian cattle sera have also
371 been tested using IDEXX Toxotest Ab Test (TXT1135T) (data in supplementary Table S1),
372 again with low concordance with MAT and overall direct detection. Moreover, a lack of
373 concordance between direct detection and presence of antibodies was observed previously in
374 studies using different detection methods (Wyss et al., 2000; Opsteegh et al., 2011b). Therefore,
375 the discordance with direct detection appears to be consistent across serological assays used for
376 indirect detection.

377 With regard to direct *T. gondii* detection, the results of the three methods used in this study did
378 not overlap. The observed discordance between the different direct detection methods may have
379 several possible explanations. Diaphragm samples were tested by MC-PCR, whereas liver
380 digests were tested by PCR and mouse bioassay, and although no consistent predilection sites
381 have been identified in cattle, it is clear that different tissues are not necessarily equally infected
382 with *T. gondii* (Opsteegh et al., 2016; Burrells et al., 2018). Liver digests were tested by PCR
383 and mouse bioassay, but no positives were identified by PCR. This is likely due to a lower
384 sensitivity of the PCR compared to mouse bioassay, as previously shown in experimental
385 infections in calves (Burrells et al., 2018), and is at least in part explained by a lower sample
386 volume. Testing a larger sample volume per animal (e.g. by increasing the number of tissues
387 tested per cattle by mouse bioassay) or the use of a more sensitive direct detection method (e.g.
388 cat bioassay) might have resulted in a higher number of direct detection positive cattle. In case
389 these additional positive cattle were also mainly seropositive, this could increase the

concordance between direct and indirect detection. However, in the current study, the lack of concordance is consistent whichever direct detection method is considered, therefore an increase in concordance is not expected.

In summary, *T. gondii* was detected in 13 out of 401 cattle and in 6 (1.6%) of 385 cattle, the detection was based on mouse bioassay, thus indicating the presence of viable parasites and a potential risk for consumers. However, the discordance between the results obtained by different direct detection methods, the lack of serological positivity in mice, and the low DNA concentrations revealed by qPCR in mouse brains and bovine diaphragms, suggest that the number of parasites in bovine liver and diaphragm is low.

The lack of concordance between MAT results and the different direct detection methods employed, indicates that MAT is unsuitable to obtain an estimate of the prevalence of viable *T. gondii* in cattle and does not provide an indication of the risk for consumers. Misclassification due to the particular direct or indirect detection methods used in the study may have influenced the concordance. However, a discordance was observed irrespective of the direct detection method, and could not be resolved by using a more conservative cut-off value for the MAT or the use of immunoblotting. For that reason, the observed discrepancy between the different methods likely represents a true lack of correlation between the presence of antibodies and the presence of (viable) *T. gondii* in cattle.

Based on these results, direct detection methods are preferred to obtain an indication of the risk for consumers from undercooked beef, preferably by implementing prevalence data into quantitative risk assessment models that take into account consumption habits. Given the performance of currently available serological methods, the implementation of serological screening for *T. gondii* to identify high risk herds or cattle at farm or slaughterhouse level is not recommended. As current direct detection methods are not feasible for the large-scale testing

414 required in case of slaughterhouse screening, the possibility to develop new methods that can
415 provide an indication of the presence of parasites should be explored.

Acknowledgements

This work was supported by the European Food Safety Authority and conducted by a consortium within the framework of project number GA/EFSA/BIOHAZ/2013/01 entitled "Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat" (€ 400.000). This publication is based on the results obtained in the framework of this mentioned project and it is published under the sole responsibility of the authors, and shall not be considered as an EFSA output. The UK component of this work was supported by the Food Standard Agency United Kingdom [project FS517004].

We are extremely grateful to Dr. Gioia Capelli, Dr. Lucia Decastelli, Dr. Fernando Fuso, Dr. Vito Perrone and Dr. Gianfranco Santagada in Italy, and Nikolaos Dadios and Phil Hardley in the UK for the organization of sample collection at the slaughterhouses; to Charlotte Cockle and Alessandra Taroda (Universidade Estadual de Londrina, Londrina, Brazil) in the UK, Andrea Bärwald in Germany, Régine Geers in France and Zsuzsa Kalmar in Romania for technical assistance.

References

Baril, L., Ancelle, T., Goulet, V., Thulliez, P., Tirard-Fleury, V., Carme, B., 1999. Risk factors for *Toxoplasma* infection in pregnancy: a case-control study in France. Scand J Infect Dis 31, 305-309.

Belluco, S., Patuzzi, I., Ricci, A., 2018. Bovine meat versus pork in *Toxoplasma gondii* transmission in Italy: A quantitative risk assessment model. Int J Food Microbiol 269, 1-11.

Belluco, S., Simonato, G., Mancin, M., Pietrobelli, M., Ricci, A., 2017. *Toxoplasma gondii* infection and food consumption: A systematic review and meta-analysis of case-controlled studies. Crit Rev Food Sci, 1-12.

Berger-Schoch, A.E., Herrmann, D.C., Schares, G., Muller, N., Bernet, D., Gottstein, B., Frey, C.F., 2011. Prevalence and genotypes of *Toxoplasma gondii* in feline faeces (oocysts) and meat from sheep, cattle and pigs in Switzerland. Vet Parasitol 177, 290-297.

Bouwknegt, M., Devleesschauwer, B., Graham, H., Robertson, L.J., van der Giessen, J.W.B., the Euro-FBP workshop participants, 2018. Prioritisation of food-borne parasites in Europe, 2016. Eurosurveillance 23, 17-00161.

Brown, L.D., Cai, T.T., DasGupta, A., 2001. Interval Estimation for a Binomial Proportion. Stat Sci 16, 101-117.

Burrells, A., Taroda, A., Opsteegh, M., Schares, G., Benavides, J., Dam-Deisz, C., Bartley, P.M., Chianini, F., Villena, I., van der Giessen, J., Innes, E.A., Katzer, F., 2018. Detection and dissemination of *Toxoplasma gondii* in experimentally infected calves, a single test does not tell the whole story. Parasit Vectors 11, 45.

Cook, A.J., Gilbert, R.E., Buffolano, W., Zufferey, J., Petersen, E., Jenum, P.A., Foulon, W., Semprini, A.E., Dunn, D.T., 2000. Sources of *Toxoplasma* infection in pregnant women: European multicentre case-control study. European Research Network on Congenital Toxoplasmosis. Bmj 321, 142-147.

Dubey, J.P., 1998. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. Parasitology 116 (Pt 1), 43-50.

Dubey, J.P., 2010. Toxoplasmosis of animals and humans, second ed. CRC Press, Florida.

Dubey, J.P., Desmonts, G., 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet J 19, 337-339.

458 Dubey, J.P., Desmonts, G., McDonald, C., Walls, K.W., 1985. Serologic evaluation of cattle inoculated
459 with *Toxoplasma gondii*: comparison of Sabin-Feldman dye test and other agglutination tests. Am J
460 Vet Res 46, 1085-1088.
461 Dubey, J.P., Hill, D.E., Jones, J.L., Hightower, A.W., Kirkland, E., Roberts, J.M., Marcet, P.L.,
462 Lehmann, T., Vianna, M.C.B., Miska, K., Sreekumar, C., Kwok, O.C.H., Shen, S.K., Gamble, H.R.,
463 2005. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the
464 United States: Risk assessment to consumers. J Parasitol 91, 1082-1093.
465 Dubey, J.P., Lindsay, D.S., Speer, C.A., 1998. Structures of *Toxoplasma gondii* tachyzoites,
466 bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11, 267-
467 299.
468 Dubey, J.P., Thulliez, P., 1993. Persistence of tissue cysts in edible tissues of cattle fed *Toxoplasma*
469 *gondii* oocysts. Am J Vet Res 54, 270-273.
470 EFSA, 2011. Technical specifications on harmonised epidemiological indicators for public health
471 hazards to be covered by meat inspection of swine. EFSA Journal 9, 2371.
472 EFSA BIOHAZ Panel, 2013. Scientific Opinion on the public health hazards to be covered by
473 inspection of meat (bovine animals). EFSA Journal 11, 3266.
474 Esteban-Redondo, I., Maley, S.W., Thomson, K., Nicoll, S., Wright, S., Buxton, D., Innes, E.A., 1999.
475 Detection of *T. gondii* in tissues of sheep and cattle following oral infection. Vet Parasitol 86, 155-171.
476 FAO, WHO, 2014. Multicriteria-based ranking for risk management of food-borne parasites,
477 Microbiological Risk Assessment Series 23, p. 287.
478 Galal, L., Schares, G., Stragier, C., Vignoles, P., Brouat, C., Cuny, T., Dubois, C., Rohart, T., Glodas,
479 C., Dardé, M.-L., Kane, M., Niang, Y., Diallo, M., Sow, A., Aubert, D., Hamidović, A., Ajzenberg, D.,
480 Mercier, A., 2018. Diversity of *Toxoplasma gondii* strains shaped by commensal communities of small
481 mammals. Int J Parasitol, https://doi.org/10.1016/j.ijpara.2018.11.004
482 Hald, T., Aspinall, W., Devleesschauwer, B., Cooke, R., Corrigan, T., Havelaar, A.H., Gibb, H.J.,
483 Torgerson, P.R., Kirk, M.D., Angulo, F.J., Lake, R.J., Speybroeck, N., Hoffmann, S., 2016. World
484 Health Organization Estimates of the Relative Contributions of Food to the Burden of Disease Due to
485 Selected Foodborne Hazards: A Structured Expert Elicitation. PLoS One 11, e0145839.

Havelaar, A.H., Kirk, M.D., Torgerson, P.R., Gibb, H.J., Hald, T., Lake, R.J., Praet, N., Bellinger, D.C., de Silva, N.R., Gargouri, N., Speybroeck, N., Cawthorne, A., Mathers, C., Stein, C., Angulo, F.J., Devleesschauwer, B., 2015. World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. PLoS Med 12, e1001923.

Homan, W.L., Vercammen, M., De Braekeleer, J., Verschueren, H., 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30, 69-75.

Hosein, S., Limon, G., Dadios, N., Guitian, J., Blake, D.P., 2016. *Toxoplasma gondii* detection in cattle: A slaughterhouse survey. Vet Parasitol 228, 126-129.

Hosseininejad, M., Azizi, H.R., Hosseini, F., Schares, G., 2009. Development of an indirect ELISA test using a purified tachyzoite surface antigen SAG1 for sero-diagnosis of canine *Toxoplasma gondii* infection. Vet Parasitol 164, 315-319.

Jones, J.L., Dargelas, V., Roberts, J., Press, C., Remington, J.S., Montoya, J.G., 2009. Risk factors for *Toxoplasma gondii* infection in the United States. Clin Infect Dis 49, 878-884.

Maksimov, P., Buschtöns, S., Herrmann, D.C., Conraths, F.J., Görlich, K., Tenter, A.M., Dubey, J.P., Nagel-Kohl, U., Thoms, B., Bötcher, L., Kühne, M., Schares, G., 2011. Serological survey and risk factors for *Toxoplasma gondii* in domestic ducks and geese in Lower Saxony, Germany. Vet Parasitol 182, 140-149.

Munday, B.L., 1978. Bovine toxoplasmosis: experimental infections. Int J Parasitol 8, 285-288.

Munday, B.L., Corbould, A., 1979. Serological responses of sheep and cattle exposed to natural *Toxoplasma* infection. Aust J Exp Biol Med Sci 57, 141-145.

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., van der Giessen, J., 2010a. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int J Food Microbiol 139, 193-201.

Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A., van der Giessen, J., 2010b. Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. Int J Food Microbiol 139, 193-201.

Opsteegh, M., Maas, M., Schares, G., van der Giessen, J., 2016. Relationship between seroprevalence

514 in the main livestock species and presence of *Toxoplasma gondii* in meat
515 (GP/EFSA/BIOHAZ/2013/01) An extensive literature review. Final report. EFSA Supporting
516 Publications 13, 996E-n/a.
517 Opsteegh, M., Prickaerts, S., Frankena, K., Evers, E.G., 2011a. A quantitative microbial risk
518 assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. Int J Food Microbiol 150,
519 103-114.
520 Opsteegh, M., Teunis, P., Zuchner, L., Koets, A., Langelaar, M., van der Giessen, J., 2011b. Low
521 predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA. Int J
522 Parasitol 41, 343-354.
523 Pardini, L., Maksimov, P., Herrmann, D.C., Bacigalupe, D., Rambeaud, M., Machuca, M., More, G.,
524 Basso, W., Schares, G., Venturini, M.C., 2012. Evaluation of an in-house TgSAG1 (P30) IgG ELISA
525 for diagnosis of naturally acquired *Toxoplasma gondii* infection in pigs. Vet Parasitol 189, 204-210.
526 Reischl, U., Bretagne, S., Kruger, D., Ernault, P., Costa, J.M., 2003. Comparison of two DNA targets
527 for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer
528 hybridization probes. BMC Infect Dis 3, 7.
529 Sabin, A., 1941. Toxoplasmic encephalitis in children. J Amer Med Assoc 116, 801-814.
530 Said, B., Halsby, K.D., O'Connor, C.M., Francis, J., Hewitt, K., Verlander, N.Q., Guy, E., Morgan, D.,
531 2017. Risk factors for acute toxoplasmosis in England and Wales. Epidemiol Infect 145, 23-29.
532 Santos, S.L., de Souza Costa, K., Gondim, L.Q., da Silva, M.S., Uzeda, R.S., Abe-Sandes, K.,
533 Gondim, L.F., 2010. Investigation of *Neospora caninum*, *Hammondia* sp., and *Toxoplasma gondii* in
534 tissues from slaughtered beef cattle in Bahia, Brazil. Parasitol Res 106, 457-461.
535 Schares, G., Bangoura, B., Randau, F., Goroll, T., Ludewig, M., Maksimov, P., Matzkeit, B., Sens, M.,
536 Bärwald, A., Conraths, F.J., Opsteegh, M., Van der Giessen, J., 2017. High seroprevalence of
537 *Toxoplasma gondii* and probability of detecting tissue cysts in backyard laying hens compared with
538 hens from large free-range farms. Int J Parasitol 47, 765-777.
539 Schares, G., Koethe, M., Bangoura, B., Geuthner, A.C., Randau, F., Ludewig, M., Maksimov, P., Sens,
540 M., Barwald, A., Conraths, F.J., Villena, I., Aubert, D., Opsteegh, M., Van der Giessen, J., 2018.
541 *Toxoplasma gondii* infections in chickens - performance of various antibody detection techniques in

542 serum and meat juice relative to bioassay and DNA detection methods. Int J Parasitol 48, 751-762.
543 Smith, J.L., 1993. Documented outbreaks of toxoplasmosis: Transmission of *Toxoplasma gondii* to
544 humans. J Food Protect 56, 630-639.
545 Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int J
546 Parasitol 30, 1217-1258.
547 Tzanidakis, N., Maksimov, P., Conraths, F.J., Kiossis, E., Brozos, C., Sotiraki, S., Schares, G., 2012.
548 *Toxoplasma gondii* in sheep and goats: Seroprevalence and potential risk factors under dairy
549 husbandry practices. Vet Parasitol 190, 340-348.
550 Villena, I., Durand, B., Aubert, D., Blaga, R., Geers, R., Thomas, M., Perret, C., Alliot, A., Escotte-
551 Binet, S., Thebault, A., Boireau, P., Halos, L., 2012. New strategy for the survey of *Toxoplasma gondii*
552 in meat for human consumption. Vet Parasitol 183, 203-208.
553 Wells, B., Shaw, H., Innocent, G., Guido, S., Hotchkiss, E., Parigi, M., Opsteegh, M., Green, J.,
554 Gillespie, S., Innes, E.A., Katzer, F., 2015. Molecular detection of *Toxoplasma gondii* in water samples
555 from Scotland and a comparison between the 529bp real-time PCR and ITS1 nested PCR. Water Res
556 87, 175-181.
557 Wyss, R., Sager, H., Muller, N., Inderbitzin, F., Konig, M., Audige, L., Gottstein, B., 2000. [The
558 occurrence of *Toxoplasma gondii* and Neospora caninum as regards meat hygiene]. Schweizer Archiv
559 fur Tierheilkunde 142, 95-108.

560 **Figure legends**

561 **Figure 1**: Number of animals by anti-*T. gondii* IgG titer using modified agglutination test

562 (MAT) in 167 calves (≤12 months) and 235 cattle (>12 months).

563

564 **Table 1:** Mean age and sex of cattle sampled in Italy (IT), the Netherlands (NL), Romania
565 (RO) and the United Kingdom (UK).

Country	Calves		Cattle	
	Number (M:F)	Mean age in months (range)	Number (M:F)	Mean age in months (range)
IT	50 (48:2)	7.3 (5-10)	49 (16:32)[a]	42.5 (13-169)
NL	50 (43:7)	6.5 (6-7)	50 (0:50)	65.6 (29-128)
RO	50 (35:15)	4.3 (0-12)	50 (6:44)	96.4 (14-225)
UK	17 (17:0)	1.24 (0-12)[b]	85 (48:37)	27.9 (13-90)
Total	167 (143:24)	5.5 (0-12)	234 (70:163)	53.7 (13-225)

566 [a]Sex was missing for 1 adult cattle from Italy (excluded from the sex distribution), and age and
567 sex were missing for one other cattle from Italy (excluded from this table).
568 [b]Sixteen calves were sampled in designated slaughterhouse for calves, these were between 0
569 and 2 months old, one 12-month old male was sampled at a slaughterhouse for adult cattle.
570

571 **Table 2:** Concordance between detection of IgG antibodies to *Toxoplasma gondii* in cattle by

572 modified agglutination test (MAT) and p30 immunoblot.

	p30 immunoblot			
MAT titer	negative	inconclusive	positive	NA[a]
<1:6	267	59	14	2
1:6 - 1:100	24	13	14	5
≥1:100	1	0	3	0

573 [a]NA: not available

574 **Table 3:** Seroprevalence of *Toxoplasma gondii* in cattle by age category and country
575 (modified agglutination test (MAT) with cut-off ≥1:6 and ≥1:100, immunoblot (IB))
576 with 95% confidence intervals ('Wilson' score interval (Brown et al., 2001)) and odds-
577 ratios (OR) by multivariable logistic regression analysis (MAT with cut-off ≥1:6).

Variable	Seroprevalence (95% CI)			OR (95% CI)	p-value LRT[a]
	MAT ≥1:6	MAT ≥1:100	IB[b]	MAT ≥1:6	
Age category					
Calves (n=167)	9.6 (6.0-15.0)	1.2 (0.3-4.3)	10.4 (6.4-16.5)	reference	0.002
Cattle (n=234)	18.8 (14.3-24.3)	0.9 (0.2-3.1)	8.9 (5.6-14.0)	2.7 (1.4-5.1)	
Country					
Italy (n=99)[c]	4.0 (1.6-9.8)	0.0 (0.0-3.7)	6.1 (2.6-13.5)	reference	<0.0005
Netherlands (n=100)	19.0 (12.5-27.8)	1.0 (0.2-5.5)	10.7 (5.5-19.7)	5.7 (1.9-17.7)	
Romania (n=100)	25.0 (17.6-34.3)	2.0 (0.6-7.0)	12.7 (7.0-21.8)	8.3 (2.7-25.0)	
UK (n=102)	11.8 (6.9-19.5)	1.0 (0.2-5.4)	9.2 (4.7-17.1)	2.4 (0.7-7.9)	

578 [a]LRT: likelihood ratio-test

579 [b]Missing (n=7) and inconclusive (n=72) IB results are excluded from the analysis

580 [c]One animal missing age and sex information was excluded from the logistic regression analysis

581

582 **Table 4:** Main features and test results for cattle positive for *Toxoplasma gondii* by a direct

583 detection method.

Country	Age (months)	Sex	Type	Positive direct detection result	Indirect detection result	
					MAT	Immunoblot
Italy	67	F	Dairy	1 mouse PCR+	negative	negative
Italy	15	F	Dairy	1 mouse PCR+	negative	negative
Italy	18	M	Beef	2 mice PCR+	negative	negative
Italy	18	M	Beef	1 mouse PCR+	negative	negative
Netherlands	107	F	NA[a]	1 mouse PCR+	1:6	inconclusive
Netherlands	128	F	NA	1 mouse PCR+	1:50	positive
Romania	154	F	Crossbreed	MC-PCR+	1:6	negative
Romania	8	M	Crossbreed	MC-PCR+	negative	negative
Romania	2	M	Crossbreed	MC-PCR+	negative	negative
Romania	12	M	Crossbreed	MC-PCR+	1:100	negative
Romania	3	F	Dairy	MC-PCR+	negative	negative
Romania	1	F	Dairy	MC-PCR+	negative	negative
Romania	10	M	Crossbreed	MC-PCR+	1:6	negative

584 [a]NA: not available

585

586 **Table 5:** Direct detection of *Toxoplasma gondii* by mouse bioassay on trypsin digested liver
587 (MBio), PCR on liver digest or MC-PCR on the diaphragm in cattle from Italy (IT), the
588 Netherlands (NL), Romania (RO) and the United Kingdom (UK), stratified by serological status
589 by modified agglutination test (MAT; cut-off ≥1:6).

Test for direct detection	MAT result	Direct detection result					
		No. of positives/no. of examined					Proportion of positives (95% CI)
		IT	NL	RO	UK	Total	
Mbio	negative	4/96	0/81	0/75	0/73	4/325	1.2% (0.4-2.9%)
	positive	0/4	2/19	0/25	0/12	2/60	3.3% (0.7-10.3%)
	total	4/100	2/100	0/100	0/85	6/385	1.6% (0.7-3.2%)
PCR digest	negative	0/96	0/81	0/75	0/89	0/341	0.0%
	positive	0/4	0/19	0/25	0/12	0/60	0.0%
	total	0/100	0/100	0/100	0/101	0/401	0.0%
MC-PCR	negative	0/26	0/24	4/20	0/22	4/92	4.3% (1.5-10.0%)
	positive	0/4	0/19	3/24[a]	0/12	3/59	5.1% (1.5-13.0%)
	total	0/30	0/43	7/44	0/34	7/151	4.6% (2.1-8.9%)

590 [a]One seropositive cattle from Romania was not tested by MC-PCR

591

Table 6: Direct detection of *Toxoplasma gondii* MC-PCR on the diaphragm in cattle, or by mouse bioassay on trypsin digested liver, stratified by serological status by modified agglutination test (MAT; cut-off ≥1:100) or p30 immunoblot, and kappa-value with 95% confidence interval (κ).

Indirect detection	Direct detection (no. positives/ no. examined (%, 95% CI))	
	MC-PCR	Mouse bioassay
MAT ≥1:100		
negative	6/147 (4.1, 1.9-8.6)	6/381(1.6, 0.7-3.4)
positive	1/4 (25.0, 4.6-70.0)	0/4 (0.0, 0.0-49.0)
total	7/151 (4.6, 2.3-9.3)	6/385 (1.6, 0.7-3.4)
	κ: 0.15 (0.01-0.29)	κ: 0
p30 immunoblot		
negative	7/92 (7.6, 3.7-14.9)	4/272 (1.5, 0.6-3.7)
doubt	0/32 (0.0, 0.0-10.7)	1/71 (1.4, 0.3-7.6)
positive	0/20 (0.0, 0.0-16.1)	1/30 (3.3, 0.6-16.7)
total	7/144	6/373
	κ: 0	κ: 0.01 (-0.12-0.14)