

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

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31 Note: Supplementary data associated with this article

32 **Abstract**

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

51

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

53 1. Introduction

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

67
68 Despite its importance as a foodborne pathogen, *T. gondii* is not covered by meat inspection.
69 Tissue cysts are of microscopic scale (Dubey et al., 1998), and meat inspection is currently
70 based on palpation, visual examination of several tissues and digestion of muscle tissues from
71 animals susceptible for *Trichinella* spp.. The European Food Safety Authority (EFSA) has
72 published scientific opinions on the feasibility of better controlling foodborne pathogens such
73 as *T. gondii* by modernization of meat inspection, for example, by using herd information to
74 classify batches into risk groups or by implementation of serological screening (e.g. (EFSA,
75 2011)). However, in case of *T. gondii* in cattle, two important gaps in knowledge exist.

76

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

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

103

104 **2. Material and Methods**

105 **2.1. Sample collection**

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

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

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

129 In each country, sample collection was performed at slaughter. At the slaughter line, the
130 investigator would select an animal for sampling when ready with labelling and storage of the
131 previous set of samples, taking into account the limit to one animal per farm and skipping
132 carcasses that were likely to be condemned. Depending on the country, between two and eight
133 slaughterhouses were visited from 2013 to 2014. Cattle were coded with a unique ID and it was
134 ensured that matching samples were labelled correctly. A minimum of 4 ml of blood was
135 collected in a 9 ml serum tube at bleeding or from the heart during evisceration. A minimum of
136 200 g of the muscular part of the diaphragm and 400 g of liver was collected in separate seal
137 bags. Age, sex and type (dairy, beef or crossbreed) of the animal were noted. Samples were
138 kept and transported on ice or in the refrigerator as much as possible. The liver was processed
139 for mouse bioassay the day after sample collection. Diaphragm samples were stored at -20 °C
140 and, if selected, sent to the National Institute for Public Health and the Environment (RIVM,
141 the Netherlands) for MC-PCR testing.

142 **2.2. Serology**

143 **2.2.1. Modified agglutination test**

144 All cattle sera were sent to ANSES – USC EpiToxo in Reims (France) and tested by a modified
145 agglutination test (MAT) as previously described (Dubey and Desmonts, 1987). Sera were
146 diluted two-fold starting at a 1:6 dilution and screened until a dilution of 1:12,800 to detect anti-
147 *T. gondii* antibodies. Both a low cut-off value of $\geq 1:6$ and a more conservative cut-off value of
148 $\geq 1:100$ (suggested by Dubey, 2010) were used to classify samples as positive or negative.

149 **2.2.2. Immunoblot using p30 antigen**

150 Additionally, cattle sera were sent to Friedrich-Loeffler-Institut (Germany) for immunoblotting
151 using affinity purified p30 antigen. To prepare antigen the RH strain of *T. gondii* (Sabin, 1941)
152 was maintained in MARC145 cell cultures and purified as previously described (Pardini et al.,
153 2012; Schares et al., 2017). Cell culture derived tachyzoites were frozen as a pellet at -80°C
154 until used for antigen purification. The *T. gondii* surface antigen p30 (TgSAG1, SRS29B) was
155 purified by affinity-chromatography using the monoclonal antibody P30/3 (ISL, UK)
156 essentially as described (Hosseininejad et al., 2009; Maksimov et al., 2011; Schares et al.,
157 2017). The immunoblot was performed as previously described (Pardini et al., 2012). Purified
158 p30 (0.5 μg) were incubated in non-reducing sample buffer (2 %[w/v] sodium dodecyl sulfate
159 (SDS), 10 %[v/v] glycerol, 62 mM TrisHCl, pH 6.8) for 1 min (94°C), separated in 12%[w/v]
160 SDS polyacrylamide minigels of 60 x 70 x 1 mm size and transferred to PVDF membranes
161 (Immobilon-P, Millipore). After the transfer, membranes were blocked using PBS-TG (PBS
162 with 0.05 % (v/v) Tween 20 (Sigma) and 2% (v/v) liquid fish gelatine (Serva, Germany)) and
163 cut into ~50 stripes and examined as described below. Bovine sera were diluted 1:100 in PBS-
164 TG. Peroxidase conjugated anti-bovine IgG (H + L) (Jackson ImmunoResearch Laboratories,
165 West Grove, PA, USA) was used diluted 1:500 in PBS-TG. As positive control we used an
166 ovine pool serum (i.e. a heterologous serum) collected from a naturally exposed sheep
167 (Tzanidakis et al., 2012). The negative control was represented by the serum of a calf born at
168 Friedrich-Loeffler-Institut which tested serologically negative for *T. gondii* by
169 immunofluorescence test (titer $<1:50$).

170 **2.3. Mouse bioassay of liver**

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

174 liver sample was grounded and mixed with 300 ml 0.25% trypsin (Sigma Aldrich, Trypsin
175 from porcine pancreas T4674) solution in 0.9% NaCl, and supplemented with antibiotics
176 (Penicillin/Streptomycin 60,000 IU/60 mg and Amoxicillin 100 mg). The mixture was
177 incubated for 1.5 hours at 37.0°C and then filtered through double layered gauze, followed by
178 centrifugation at 1800g for 10 minutes. The supernatant was removed, and the pellet was
179 washed in 0.9% NaCl. Afterwards, 1 ml of the pellet was kept and stored at -20°C for PCR
180 and the remaining pellet was mixed with 1 ml antibiotics solutions
181 (penicillin/streptomycin/amoxicillin and ciprofloxacin/cefotaxime/ vancomycin) and made up
182 to 5 ml total volume with 0.9% NaCl. Per sample, two mice (Swiss Webster of minimum 6
183 weeks old) per sample were inoculated intraperitoneally with 1 ml of this suspension.
184 Mice were monitored twice daily and given a health score based on coat condition and
185 demeanor. Mice were euthanized based predetermined humane endpoints. Ethical approval
186 for the mice experiments was obtained in the respective countries (details provided in section
187 2.7). The development of antibodies against *T. gondii* in mice was determined by serology on
188 day 28 in Italy (to prepare for possible strain isolation at day 42) and at post mortem (day 42)
189 in the Netherlands, Romania and the United Kingdom. Mouse sera from the Netherlands and
190 the United Kingdom were tested by ID.Vet ELISA (ID Screen® toxoplasmosis indirect multi-
191 species) and, in case of doubtful results, sent to Reims for confirmation by MAT at 1:25
192 dilution; all mouse sera from Romania were sent to Reims for MAT without any prior testing;
193 sera from Italy were tested by Toxoscreen DA (BioMerieux). For PCR-based detection in
194 mice, at least half of the mouse brain was homogenized in PBS (75 µl PBS per 25 mg of
195 tissue) and 100 µl of this homogenate was used for DNA isolation as described in the
196 manufacturer's protocol for human or animal tissue and cultured cells (NucleoSpin Tissue,
197 Macherey-Nagel, Germany). The presence of *T. gondii* DNA was investigated by PCR, either
198 qPCR targeting a 529 bp Repeated Element (RE) (Wells et al., 2015) or, in Romania, by

199 conventional PCR on the 529 bp RE followed by gel electrophoresis (Homan et al., 2000). To
200 consider a mouse brain as qPCR positive, all negative and blank controls in the same PCR run
201 had to be negative, and the C_q-value for the sample had to be <40 with the shape of the
202 amplification curve similar to those of the positive controls. If so, samples with a C_q-value
203 <35 were considered positive, samples with a C_q-value between 35 and 40 were additionally
204 confirmed by identification of the correct band size (162 bp) by gel electrophoresis.
205 Conventional PCR was considered positive when an amplicon of the expected size (529 bp)
206 was identified by gel electrophoresis. A mouse bioassay was considered positive if at least
207 one of the two mice was positive by serology or PCR.

208 **2.4. PCR on liver digest**

209 DNA was isolated from liver digests using the Nucleospin kit (Machery-Nagel) as follows: 200
210 µl of digest, 1440 µl of T1 buffer and 200 µl of proteinase K (provided with the kit) were mixed
211 and incubated at 56 °C for 1-3 hours. After incubation, 230 µl of the mixture was used for
212 further processing to prevent overloading of the column. The manufacturer's protocol for
213 human or animal tissue or cultured cells was followed as described from step 3 onward (adding
214 200 µl of B3). Samples were subsequently tested by 529 bp RE qPCR (Wells et al., 2015) or
215 by conventional PCR on the 529 bp RE (Homan et al., 2000). PCR screening of liver digests
216 was performed in the country of sample collection. PCR was considered positive using the
217 criteria described for PCR on mouse brains.

218 **2.5. MC-PCR on diaphragm**

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

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

226 **2.6. Data analysis**

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

235 **2.7. Ethical approval**

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

241 The *in vivo* protocol was approved by the Italian Ministry of Health (artt. 8 and 9, D.L.vo
242 116/92, 5th December 2013); by the Animal Ethics Committee of the Animal Sciences Group
243 (Lelystad, the Netherlands) (2014090.d, 24th November 2014); by the Animal Ethics
244 Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca,
245 Romania (30/2015 USAMV CN); and by the Moredun Experiments and Ethical Review
246 Committee, United Kingdom (E51/14, 30th September 2014).

247 **3. Results**

248 **3.1. Features of the samples**

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

255 **3.2. Detection of anti-*T. gondii* IgG**

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

263 **3.3. Direct detection of *T. gondii***

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

270 **3.3.1. Mouse bioassay**

271 Six out of 385 (1.6%) cattle tested positive by mouse bioassay (Table 5). Mice were tested
272 serologically and by PCR on the brain, and if either of these methods gave a positive result in
273 one of the two mice inoculated with the same liver digest, the mouse bioassay was scored
274 positive. For one adult cow from the UK no liver sample was collected, and for sixteen calves
275 (<2 months) from the UK, no analysis could be made by mouse bioassay. For 22 cattle the
276 mouse bioassay results were incomplete (e.g. a serological result would be missing when a
277 mouse had to be excluded prior to the end of the bioassay). These 22 cattle were considered
278 mouse bioassay negative based on the available results. With the exception of one cattle liver
279 from Italy, in which both mice of the bioassay tested positive by qPCR on the brain, positive
280 mouse bioassays were based on a positive PCR result for one mouse with C_q-values ranging
281 from 34 to 39. All positive and negative controls in DNA isolation and PCR gave appropriate
282 results.

283 **3.3.2. PCR on liver digest**

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

286 **3.3.3. MC-PCR on diaphragm**

287 MC-PCR targeting the 529 bp RE of *T. gondii* was performed on diaphragms from 151 cattle.
288 Twenty-five cattle per country were tested irrespective of the results by MAT. In addition,
289 diaphragms from all cattle positive by mouse bioassay, liver digest PCR or MAT, were tested,
290 with the exception of one Romanian cattle with a MAT titer of 1:6, which was omitted by
291 mistake. Seven cattle out of 151 (4.6%), gave a positive MC-PCR result (Table 5) with C_q-
292 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 \geq 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 (\geq 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 were 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.

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

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

362 The concordance between direct and indirect detection can be influenced by the choice of
363 detection methods, as recently shown in chickens (Schaes et al., 2018). In the current study,
364 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

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

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

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

408 Based on these results, direct detection methods are preferred to obtain an indication of the risk
409 for consumers from undercooked beef, preferably by implementing prevalence data into
410 quantitative risk assessment models that take into account consumption habits. Given the
411 performance of currently available serological methods, the implementation of serological
412 screening for *T. gondii* to identify high risk herds or cattle at farm or slaughterhouse level is not
413 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.

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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 ^aSex 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 ^bSixteen 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.

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

573 ^aNA: not available

574 **Table 3:** Seroprevalence of *Toxoplasma gondii* in cattle by age category and country
575 (modified agglutination test (MAT) with cut-off $\geq 1:6$ and $\geq 1:100$, 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 $\geq 1:6$).

Variable	Seroprevalence (95% CI)			OR (95% CI) MAT $\geq 1:6$	p-value LRT ^a
	MAT $\geq 1:6$	MAT $\geq 1:100$	IB ^b		
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 ^aLRT: likelihood ratio-test

579 ^bMissing (n=7) and inconclusive (n=72) IB results are excluded from the analysis

580 ^cOne 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 ^aNA: 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 $\geq 1:6$).

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 ^aOne seropositive cattle from Romania was not tested by MC-PCR

591

592 **Table 6:** Direct detection of *Toxoplasma gondii* MC-PCR on the diaphragm in cattle, or
 593 by mouse bioassay on trypsin digested liver, stratified by serological status by modified
 594 agglutination test (MAT; cut-off $\geq 1:100$) or p30 immunoblot, and kappa-value with
 595 95% confidence interval (κ).

Indirect detection	Direct detection (no. positives/ no. examined (%), 95% CI)	
	MC-PCR	Mouse bioassay
MAT $\geq 1:100$		
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) κ : 0.15 (0.01-0.29)	6/385 (1.6, 0.7-3.4) 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 κ : 0	6/373 κ : 0.01 (-0.12-0.14)

596