Toxoplasma gondii detection in cattle: A slaughterhouse survey

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Abstract

Toxoplasmosis is a zoonotic disease caused by the protozoan parasite *Toxoplasma gondii*. Ingestion of raw or undercooked meat containing viable cysts has been suggested to be a major source of *T. gondii* infection in humans. Suboptimal performance of serological assays in cattle has traditionally precluded accurate quantification of the extent to which cattle populations are infected and their meat harbour tissue cysts. In the absence of accurate estimates of the level of infection in the animal population, assessments of likely human exposure through the consumption of cattle meat remain highly speculative. Following the development of novel and sensitive molecular methods that can be applied to the relatively large numbers of samples required in observational studies, the first quantitative estimates of the frequency of *T. gondii* in meat samples from naturally infected cattle have become available recently. Such estimates are critical for the development of quantitative risk assessment models that could be used to inform food safety policies. The aim of this study was to generate the first estimates of the prevalence of *T. gondii* infection in a sample of cattle exposed to natural levels of infection and slaughtered for human consumption in the UK under commercial conditions. Such estimates provide great value to the global assessment of *T. gondii* burden given the scarcity of data available on the frequency of natural infection in cattle populations worldwide.

Between October 2015 and January 2016 diaphragm samples were collected from 305 animals, slaughtered in ten commercial slaughterhouses across the UK. Movement histories showed that the animals sampled (41.6% females and 58.4% males) had passed through a total of 614 farms and 40 livestock markets across the country. Five animals (1.6%) were deemed positive for *T. gondii* following magnetic capture real-time PCR, confirmed by amplicon sequencing. The true prevalence of infection was estimated to be 1.79%. All positive animals were male, none of whom had been on the same farm and/or livestock market before slaughter and there was no apparent geographic pattern. The results from this study suggest a low level of infection in cattle raised and slaughtered in the UK and can be used to populate the first stages of formal risk assessments to quantify the likely extent of human exposure to *T. gondii* through the consumption of beef with relevance to the UK, EU and rest of the world.

Key words: *Toxoplasma gondii*, cattle, MC-PCR, prevalence, food safety, United Kingdom

Highlights

- Estimation of *T. gondii* prevalence in naturally infected cattle using MC-PCR
- Low level of *T. gondii* infection without geographic pattern for positive animals
- Fills gaps as a prelude to risk assessment of human foodborne exposure to *T. gondii*

1. Introduction

Toxoplasmosis is a zoonotic disease caused by the apicomplexan parasite *Toxoplasma gondii* (Montoya and Liesenfeld, 2004). Domestic cats and other felids are the definitive hosts, while mammals and birds are the most common intermediate hosts. Oocysts produced in the definitive host are passed in faeces and sporulate in the environment before being ingested by an intermediate or another definitive host. When sporulated oocysts are ingested by an intermediate host sporozoites are released, infecting numerous tissues (predilection tissues), where they undergo endodyogeny to form tachyzoites. While predilection tissues vary between species, muscle, liver, brain and the intestinal epithelium are commonly infected (Dubey et al., 1998; Roberts and Janovy, 2005). Following infection, the parasite develops into tissue cysts where the parasite multiplies (termed bradyzoites at this stage).

The sero-prevalence of *T. gondii* infection varies between host species and country. It is estimated that up to 30% of the global human population is infected (Tenter et al., 2000). Ingestion of raw or undercooked meat containing viable cysts has been suggested to be a major source of *T. gondii* infection in some European countries (Cook et al., 2000; Flatt and Shetty, 2012), however the relative contribution of different types of meats to human *T. gondii* infection is unclear. Herbivorous livestock are most likely to become infected from the ingestion of infective oocysts in the pasture, feed or drinking water (Andreoletti et al., 2007). In cattle, sero-prevalence estimates vary from 1% to 92% worldwide, however the results are not directly comparable given differences in study design and the test used. Crucially, sero-prevalence is indicative of exposure to the parasite, not cyst development. Contrary to sheep and goats, clinical signs are rarely exhibited in cattle (Dubey, 2010).

Reliable prevalence estimates in meat-producing animals are needed as the first stage in formal risk assessments aiming to estimate the relative contribution of meat to human *T. gondii* infection. The lack of information regarding the level of infection in cattle reared in the UK and Europe has been highlighted by the UK Food Standards Agency (FSA) and the European Food Safety Authority (EFSA) (AMCSF, 2012; Andreoletti et al., 2007). Routine detection of *T. gondii* cysts during meat inspection is not feasible given the microscopic size of the cysts. Instead, diagnosis of *Toxoplasma* infection most commonly relies on serological detection. Although numerous techniques are available

for detection of antibodies, the lack of correlation between seropositivity in cattle and presence of detectable cysts has limited the value of serology as an indirect indicator for cyst occurrence in beef (Opsteegh et al., 2016b; Opsteegh et al., 2011). Therefore, direct detection methods are necessary to provide estimates of the proportion of cattle harbouring cysts, a critical input for a sound assessment of the risk of human infection associated with the consumption of cattle meat.

The gold standard for detecting *T. gondii* in meat samples is bioassay using mice or cats. However, these methods are relatively expensive, very time consuming and not conducive for the screening of large sample numbers (da Silva and Langoni, 2001). More recently, molecular approaches such as polymerase chain reaction (PCR) based methods have been favoured for the detection of *T. gondii*, however PCR methods lack sensitivity when compared to the bioassay (da Silva and Langoni, 2001; Garcia et al., 2006; Hill et al., 2006). In response, a highly sensitive magnetic capture PCR- method was developed (Opsteegh et al., 2010). The method combines homogenization of a meat sample (100 grams) with sequence specific magnetic capture followed by quantitative real time PCR (qPCR) (Opsteegh et al., 2010). Using this method, we aimed to assess the level of *T. gondii* infection in cattle raised and slaughtered in the UK for human consumption. It is expected that the results of this study can inform future probabilistic assessments of the risk of human infection associated with beef consumption.

2. Material and Methods

2.1. Study design

A slaughterhouse-based study was conducted in the UK between October 2015 and January 2016. All slaughterhouses across the UK were invited to take part in the study. Ten of them showed willingness to participate and were included. Thus, the selection of slaughterhouses to be included in this study was non-probabilistic and based on voluntary participation. Each slaughterhouse was visited during two or three days, during which one animal was selected for sampling from each farm sending animals to the slaughterhouse during these days. In the case of animals coming in batches brought from livestock markets, the farm where the animal was located before going to market was considered as the farm of origin. The first animal of the batch was sampled, if the first animal of the

batch was missed, the second animal was sampled. The target number of animals was 300 for an expected prevalence of 2.9%, 95% confidence interval and 1.9% precision.

The study received ethical approval from the Royal Veterinary College Ethics and Welfare Committee under the reference URN 2015-1407.

2.2. Sample and data collection

A minimum of ~150 g of diaphragm muscle was collected at post mortem from the selected animals. Knives were rinsed and kept in hot water in between diaphragm sampling. Diaphragm samples were placed in polythene bags labelled with a unique ID and sealed to avoid leakage. ID and ear tag numbers from animals sampled were recorded in a standardised recording sheet. Samples were kept and transported on ice and stored at -20°C until ready for use. Ear tag numbers were used to obtain movement history, age, sex and breed of each animal sampled using the British Cattle Movement System (www.bcms.gov.uk).

2.3. Laboratory analysis

All oligonucleotides used for sequence specific magnetic capture and qPCR were used as designed previously by Ospteegh and colleagues (Opsteegh et al., 2010), targeting the 529-bp DNA fragment (GenBank accession number AF146527). A competitive internal amplification control (CIAC) was included as described by Ospteegh and colleagues to allow detection of false-negative PCR results (Hoorfar et al., 2004; Opsteegh et al., 2010).

Bovine diaphragm sample preparation and sequence specific magnetic capture was performed as detailed elsewhere (Opsteegh et al., 2010). In between samples, scissors and forceps were rinsed in soap and hot water and then treated with DNAzap (Ambion, Texas, USA) to minimise the risk of cross-contamination. In addition, each filter bag and contents was placed in a second plain Stomacher400 bag during homogenisation to minimise the risk of spillage.

PCR amplification was performed in 96 well plates using the BioRad CFX96 Real time detection system (Bio-Rad laboratories, CA, USA) and SsoAdvanced Universal Probes Supermix (Bio-Rad, CA, USA) in 20 µl reaction volumes per well. Each reaction consisted of 0.7 µM of Tox-9F and Tox-

11R, 0.1 µM of Tox-TP1, 0.2 µM of CIAC probe, 0.02 fg of CIAC (kindly provided by M. Opsteegh, as described previously) and 10 µl of template DNA. Cycling conditions were created according to the Supermix manufacturer's recommendations for optimized cycling and comprised of 95°C for two minutes, followed by 45 cycles 95°C for 15 seconds and 60°C for 30 seconds. On each plate, a standard series of *T. gondii* DNA was included (ranging from 5×10^7 to 5×10^1). Each reaction was carried out in duplicate and nuclease free water was used in place of DNA in quadruplicate as the non-template (negative) control. The quantification cycle value (Cq) and melt curve was used to determine the *T. gondii* status of all samples. All samples without a Cq value but positive CIAC-PCR were scored negative. Samples with no Cq value for the CIAC-PCR were repeated. Samples with a Cq value for both the *T. gondii* and CIAC assays were scored positive for *Toxoplasma* DNA presence. Samples scored as putative positives were confirmed by standard PCR using magnetically captured DNA as template with primers Tox-9F and Tox-11R (Opsteegh et al., 2010) (Bioline *Taq* polymerase, conditions as described by the manufacturer, 1 x 94 °C for 1 min, 45 x [94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s], 1 x 72 °C for 7 min). Amplicons were cloned using pGEM-T Easy (Promega) in XL1-Blue MRF *Escherichia coli* (Stratagene), miniprepped (Qiagen) and sequenced (GATC Biotech) as described by the respective manufacturers. Sequence assembly and annotation by BLASTn comparison of homology to the GenBank NR database was undertaken using CLC Main Workbench v6.0.2 (CLC Bio).

2.4. Data analysis

Descriptive statistics were obtained using R 3.0 (R Development Core Team, 2015). True prevalence was estimated stochastically in @Risk version 6 for Excel (Palisade Corporation, Newfield, NY) using sensitivity and specificity values previously reported for the test used (Se = 89.2%; 95% CI 79.2–99.2%; Sp=100%) (Opsteegh et al., 2010) and describing uncertainty in the sensitivity values by means of a pert distribution.

3. Results and Discussion

Samples were collected from 10 slaughterhouses, nine located across England and one in Scotland. Diaphragm samples from 305 animals were collected and tested using magnetic capture and qPCR; 127 (41.6%) were female between 11.3 and 147.0 months (median 26.2 months) and 178 (58.4%) were male between 9.0 and 36.7 months (median 23.2 months). Samples collected represented 34 different breeds or cross breeds (Supplementary material Table S.1). The number of sites an animal stayed before slaughter ranged from one to 15 (median 3). Overall the 305 animals sampled covered 614 different farms and 40 livestock markets across the country. The location of the farms and livestock markets, aggregated by region, is presented in Table 1.

Five (1.6%) samples were deemed positive following magnetic capture and qPCR. Average Cq values obtained from qPCR for each sample ranged from 19.36 to 41.23 in all positive animals (overall average ± standard deviation was 25.31 ± 9.31; Table 2). Since two of the five putative positives presented Cq values in excess of 40 all results were verified by independent PCR, amplicon sequencing and BLASTn annotation, confirming *T. gondii* 529-bp repeat sequence identity. Once adjusted for the test sensitivity, the mean true prevalence was estimated to be 1.79% (5^{th} and 95^{th} percentiles 1.66 and 1.95 respectively). Positive animals were slaughtered in four different slaughterhouses and none of them stayed in the same site (farm or livestock market) before being slaughtered. Although extrapolations should be made with caution given the non-probabilistic selection of animals, this study suggests a low level of infection in cattle raised and slaughtered in the UK for human consumption, with no clear geographic pattern of positive animals. When interpreting our results it is important to bear in mind that *T. gondii* positive status was defined based on testing a fraction (100g) of one elective tissue (diaphragm). It is therefore possible that some animals with cysts were deemed negative due to the absence of enough *T. gondii* DNA in the sample collected, supported by average Cq values from two of five positive animals >40.

Evidence has built up in recent years regarding the lack of concordance between the presence of antibodies in cattle and *T. gondii* cysts in beef, limiting the use of serology assays as an indirect indicator for the presence of cysts in beef (Opsteegh et al., 2016b; Opsteegh et al., 2011). Magnetic capture PCR (MC-PCR) has been reported to have improved sensitivity for detection of *T. gondii* compared with other molecular methods (Juránková et al., 2014; Opsteegh et al., 2010). In addition,

MC-PCR is considerably quicker and more cost effective that the bioassay (Opsteegh et al., 2010), and can reduce the requirement for laboratory animal use in line with the NC3Rs reduction/replacement principles. MC-PCR has successfully been used to look at the occurrence of *T. gondii* in different meat-producing animals (Hamilton et al., 2015; Juránková et al., 2014; Opsteegh et al., 2010; Opsteegh et al., 2016a). Nonetheless, MC-PCR still presents some limitations. The process is time consuming and is not directly amenable to upscaling. A relatively large tissue sample is required from each animal, limiting the choice of tissue cuts available for routine testing where material would otherwise enter the food chain.

All positive samples in this study came from male cattle aged between 15.3 and 31.2 months at the time of slaughter (Table 2). The potential effect of age on *T. gondii* infection in cattle is not clear and the scattered results available are inconsistent. A study conducted in Switzerland found a higher proportion of positive calves compared to older groups as detected by PCR using one gram of diaphragm (Berger-Schoch et al., 2011); whilst the contrary was found in a study carried out in Iran (Azizi et al., 2014). In a recent study involving four European countries no clear age pattern was found using MC-PCR on 100g of diaphragm, with the age of positive animals ranging from 1 month to 12.8 years (Opsteegh et al., 2016b). If infected cattle remain infected for life or somehow overcome the infection and eliminate the parasite over time remains unknown. All positive animals in this study were male. One previous study reported the sex of positive animals and a similar number of positive male and females were found (Opsteegh et al., 2016b). Berger-Schoch and colleges found a higher proportion of females were positive (Berger-Schoch et al., 2011). Within the same study calves were found to include the highest proportion of positive animals, but calf sex was not reported. Further research is needed to investigate the potential effect of sex and age (if any) on the presence of cysts in infected cattle before any significant conclusion can be drawn.

Toxoplasma gondii presents a complex life cycle with multiple routes of transmission and the severity of disease associated with this parasite prompts concern for human health. Earlier reports suggested a minimal risk of transmission from beef (or beef products) to humans. This was based on discrepancies between sero-prevalence in cattle and presence of infectious parasites in beef (Dubey, 1986; Dubey and Beattie, 1988). However, consumption of undercooked beef has been reported as a

risk factor for *T. gondii* infection in pregnant women in various European countries and the USA (Baril et al., 1999; Cook et al., 2000; Jones et al., 2009). Beyond parasite occurrence in meat-producing animals, the risk of human infection is also known to vary by regional and cultural habits. However, a formal risk assessment is needed in order to estimate the relative contribution of beef and other animal products to human *T. gondii* infection, and assess the potential effect of risk mitigation measures along the beef chain on reducing human exposure to *T. gondii*.

The results from this study can be used to populate the first stages of formal risk assessments as well as inform study design and sample size calculation in future studies, filling some of the data gaps previously identified by FSA (AMCSF, 2012). Given the scarcity of data available on the frequency of natural infection in cattle populations worldwide, the relevance of the results presented here goes beyond the study area and could prove of value in the EU and further afield.

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241 Table 1. Number and percentage of livestock markets and farms, per region, where sampled cattle
242 stayed.

Region	Num. of livestock markets (%)	Num. of farms (%)
England		
•South East	4 (10%)	63 (10.3%)
•South West	10 (25%)	158 (25.7%)
•East England	-	14 (2.3%)
•Greater London	-	-
•East Midlands	3 (7.5%)	72 (11.7%)
•West Midlands	8 (20%)	94(15.3%)
•Yorkshire and the Humber	1 (2.5%)	52 (8.5%)
•Northwest	2 (5%)	45 (7.3%)
•Northeast	-	8 (1.3%)
Scotland	6 (15%)	44 (7.2%)
Wales	6 (15%)	64(10.4%)
Total	40	614

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247 Table 2 Characteristics of cattle that tested positive to *Toxoplasma gondii* using MC-qPCR.

Animal ID	**Sex**	**Breed**	**Age (months)**	**Number of sites stayed**	**Site location(s)**	**qPCR (Cq)**
C006	Male	Limousin	18.1	5 (3 farms; 2 livestock markets)	Southeast and East Midlands	40.04
C172	Male	Charolais	31.2	3 (3 farms)	Northeast and Yorkshire and the Humber	20.32
C201	Male	Simmental cross	25.3	3 (2 farms; 1 livestock market)	Southwest	41.23
C278	Male	Limousin	15.3	3 (2 farms; 1 livestock market)	Scotland	19.59
C289	Male	Charolais cross	19.7	3 (2 farms; 1 livestock market)	Scotland	19.36

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