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Toxoplasma gondii: Level of exposure in pigs and cattle in the UK and a hypothetical model for human exposure

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EXECUTIVE SUMMARY

Toxoplasmosis is a zoonotic disease with a worldwide distribution caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*). The life cycle of *T. gondii* includes felines as the definitive host and mammals and birds as 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, where they undergo endodyogeny to form tachyzoites. Within the infected tissues the parasite develops into tissue cysts where the parasite multiplies (termed bradyzoites). The number of bradyzoites within a tissue cyst varies depending on the age of the cyst. Although humans can become infected through different routes, ingestion of raw or undercooked meat containing viable cysts has been suggested to be a major source of *T. gondii* infection in Europe and North America. However, the frequency and infectivity of *T. gondii* cysts in meat and how this can be inferred from the serological status of animals has been identified as an important knowledge gap by the European Food Safety Authority (EFSA). In the UK, the lack of information regarding level of infection and potential risk factors associated with *T. gondii* infection in meat-producing animals has been highlighted by the Food Standards Agency's Advisory Committee on the Microbiological Safety of Food. In order to generate data on knowledge gaps previously identified and to increase the evidence base for meat-safety decision making in relation to *T. gondii*, the Food Standards Agency (FSA) collaborated with registered organisations under article 36 of Regulation (EC) 178/2002 in a consortium involving 8 European countries and led by the Dutch National Institute of Public Health and the Environment to investigate the "*Relationship between sero-prevalence in the main livestock species and presence of Toxoplasma gondii in meat*". In the UK, FSA funded studies to be carried out by the Royal Veterinary College (RVC) in collaboration with Moredun Research Institute to conduct observational and experimental studies in cattle and pigs.

The report presents all the project components carried out in the UK with the support of the FSA. Some of these components were conducted as part of the EFSA consortium while others were carried out independently and not as part of the consortium. Where appropriate, this report refers to results generated by the EFSA consortium which are detailed in the following reports:

- *"Relationship between seroprevalence in the main livestock species and presence of Toxoplasma gondii in meat (GP/EFSA/BIOHAZ/2013/01): An extensive literature review"*
- *"Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report"*

The project components carried out in the UK with the support of the FSA are listed below; components i, ii.a, ii.b and iii were carried out as part of the EFSA consortium while components ii.c, iv and v were carried out independently of the EFSA consortium.

i. Participation in the *Extensive literature reviews* regarding (i) anatomical distribution of *T. gondii* cysts in meat and other edible tissues; (ii) available methods for detecting the presence and infectivity of *T. gondii* cysts; (iii) the relationship between sero-prevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues and (iv) on-farm risk factors for *T. gondii* infection in the main livestock species. The main findings are presented in this report.

ii. Studies in cattle:

a. A slaughterhouse-based study to assess the correlation between indirect and direct detection of *T. gondii* in cattle in four countries: The Netherlands (NL), United Kingdom (UK), Romania (RO), and Italy (IT).

b. An experimental study to determine the dissemination of *T. gondii* cysts to different tissues, organs and cuts of meat intended for the food chain following infection with *T. gondii* oocysts. The experimental infection with *T. gondii* in calves was carried out at the Moredun Research Institute

c. Abattoir survey to assess the level of infection by *T. gondii* in cattle in the UK.

iii. Studies in pigs:

- A cross sectional study to assess the level of exposure to *T. gondii* in pigs raised in commercial farms in England and identify factors associated with increased risk of exposure.

iv. An in-depth review of three previous risk assessments and relative contribution of different animal products to human *T. gondii* infection. The review highlights key assumptions, outputs and data gaps identified in each risk assessment.

v. The development of a hypothetical stochastic model to assess human exposure to *T. gondii* through consumption of meat products. The model can be used to generate the probability distribution describing the occurrence of viable cysts in edible tissues. The steps in this assessment for which data are not currently available are highlighted and the implications on the results are discussed.

The main findings from these five project components are summarized below.

i. *Extensive literature review*. The first step of the EFSA project was a review of the literature which gathered available evidence in four main areas of concern:
    - **Anatomical distribution of the cysts in meat and other edible tissues**: brain and heart were predilection sites repeatedly identified in pigs, sheep, goats, poultry and horses.
    - **Available methods for detecting the presence and infectivity of *T. gondii* cysts**: Mouse and cat bioassay and PCR-based methods were the main methods reported as capable of detecting cysts in meat. Using data from papers that presented matched results using two or more direct detection methods, cat bioassay detected more positive animals in more studies, followed by mouse bioassay. Most PCR-based methods can perform similarly to the mouse bioassay depending on sampling protocols, however PCR methods do not demonstrate infectivity of the parasite. The size of the sample was identified as an important factor that influences the ability of the test to detect cysts; suggesting that the distribution of infective cysts is uneven, and therefore the larger the sample tested, the higher the likelihood that an infected tissue is detected as positive. Notably there were no studies that quantified the amount of *T. gondii* bradyzoites in tissue cysts.
    - **Relationship between sero-prevalence in the main livestock species and presence of *T. gondii* cysts in their meat:** overall, a fair to moderate level of concordance was reported in pigs, small ruminants and chickens between detection of antibodies to *T. gondii* and direct detection of the parasite in meat. On the contrary, the limited data available on cattle and horses suggest a lack of concordance between serology and presence of *T. gondii* in these two species.
    - **On-farm risk factors for *T. gondii* infection in the main livestock species**: presence of the definitive host (cats) and faecal contamination by cats increased the risk of animals being *T. gondii* positive in all livestock species. Level of confinement, water source and size of the herd produced contradictory results across studies, and the role they can have on the risk of *T. gondii* infection was not clear.

ii. Studies in cattle

a. Cattle (n=402) from commercial slaughterhouses in four countries were included in a multi-country study aimed at assessing the correlation between indirect and direct detection of *T. gondii*. Only 13 cattle (3.2%) were considered positive using a direct detection method (MC-PCR or bioassay) and none of these animals came from the UK. Out of these 13 positive animals only 5 were serologically positive, while 55 animals tested positive by serology (12 of them from the UK) and were negative to MC-PCR and/or Bioassay. Results from this study support literature review findings regarding the lack of agreement between serology and the presence of cysts. Hence, the results suggest that serology should not be used in cattle as a proxy for presence of viable *T. gondii* in beef.

b. The experimental study to determine the dissemination of *T. gondii* cysts to different tissues, organs and cuts of in cattle included 6 calves all of which, following oral infection with $1x10^6$ *T. gondii* oocysts became seropositive by modified agglutination test (MAT) by 21-days post infection. Both viable *T. gondii* and DNA were detected in various tissues including meat cuts, no clear predilection sites could be identified.

c. Based on the main findings from the literature reviews and the multi-country study and calf experimental infections, an abattoir study aimed at generating information on the level of *T. gondii* infection in cattle slaughtered for human consumption in the UK was conducted. Diaphragm samples were collected from 305 animals slaughtered in commercial slaughterhouses in the UK and movement history of animals sampled was obtained through the British cattle movement system (BCMS). Animals sampled (127 females and 178 males) had been exposed to a total of 614 different farms and 40 livestock markets across the country. Five animals (1.6%) were deemed positive following analysis with MC-PCR. All positive animals were male and none of them had been in the same farm and/or livestock market before slaughter. Cq values of animals presume positive animals ranged from 17.03 to 44.04 suggesting some variation on the parasite load of positive animals. The results from this study suggest a low level of infection in cattle raised and slaughtered in the UK and no clear geographic pattern of positive cases emerged.

iii. *Cross sectional study in pigs:* A cross sectional study was conducted to assess the level of exposure to *T. gondii* in pigs raised in England, to identify factors associated with a higher risk of exposure gathered by means of a standardised questionnaire and to compare the performance of two commercially available serological tests (Modified agglutination tests for the detection of *T gondii* specific immunoglobulin-IgG (MAT) and enzyme-linked immunosorbent assays multispecies (ELISA)). The main findings from the literature review were used to develop the study protocol and questionnaire

- A total of 2071 pigs at slaughter originating from 131 farms were sampled. A low proportion of pigs tested positive to MAT (3.6%) with the majority of the pigs having a low MAT titre, suggesting a low level of exposure to *T. gondii* in the farms studied. Crucially, most of the positive pigs came from a small number of farms suggesting that risk of *T. gondii* infection in pigs is largely driven by farm-level factors.
- A subset of sera samples (n=492) were tested using a commercially available ELISA. A moderate agreement (Kappa=0.61; 95%Ci 0.52-0.70) was found for results obtained by MAT and ELISA. Overall, more samples were classified as positive using MAT.
- A Bayesian model was used estimate the farm-level prevalence and the probability of each farm having at least one positive animal by MAT. Once adjusted for the number of animals tested per batch and the Sensitivity and Specificity of the test, the estimated herd level prevalence for the farms sampled was 11.5% (95% credibility 8.4%-16.0%), with most of the positive pigs coming from a small number of farms. Although this is a sample estimate and not a population estimate, the level of exposure to *T. gondii* in commercial pigs appears to be lower than those reported in Germany, Serbia, Italy, Spain and Greece. However, comparisons should be made with caution given differences in study designs. The results from this study suggest a low level of exposure to *T. gondii* in the farms studied, most of which are likely to send to slaughter batches composed of 100% uninfected pigs.
- Seventy three farms returned a completed questionnaire and were included in the risk factors analysis. Farms were classified as positive or negative using two cut offs for positive farms: those for which the probability of having at least one true positive pig was ≥0.50 (n=5, 6.8%) and those for which the probability was ≥0.10 (n=13, 17.8%). There was no statistically significant association ($p \leq 0.05$)

between positive status and any of the putative risk factors explored when a 50% cut off was considered. However, some patterns did arise: the presence of cats (either belonging to the farm or coming from outside) increased the risk 2.5 fold, whether cats could access pigs' food increased the risk by 2.6 and having an open feed storage increased the risk of positive status by 5.8, suggesting that the presence of cats and the unnoticed feed contamination with cat's faeces are both important. Using a 10% cut off, the relative risk (RR) of infection was higher on those farms that allow outdoor access of pigs at any production stage (RR=3; p=0.04), those farms keeping ≤200pigs (RR=3.9; p=0.02) and farms where cats have direct access to pigs' feed (RR=2.6; p=0.04). Based on these results, recommendations to farmers should emphasise the importance of ensuring that cats do not have access to pigs feed in order to avoid food contamination. Such recommendations should reduce the level of exposure to sporulated oocysts and therefore, the level of infection regardless of the herd size and level of confinement.

iv. *Review of previous reported risk assessments*. Decisions with regard to management of the risk of *T. gondii* infection associated to meat consumption should be based on scientific risk assessment. The results from the project components outlined above have helped to improve knowledge regarding key data gaps that were previously identified, specifically: (i) evidence was generated as part of the EFSA project on the concordance between serological status and presence of viable cysts in the main livestock species. Crucially, evidence was generated in those species where limited data were previously available (cattle and horses); and (ii) evidence was generated on predilection sites for infective cysts and the potential effect of vaccination on viable cysts in sheep and pigs. Specifically in the UK, the abattoir cattle studies and the cross sectional study in pigs provided the first approximation to the level of exposure of these two species to *T. gondii*. The results from these studies can be used to inform study design and sample size calculation in future studies and monitoring activities and could be used to populate formal risk assessments. Such assessments require a number of inputs some of which may be supported by limited data. Key knowledge gaps that are essential for a sound quantitative assessment of risk of human exposure to *T. gondii* through meat still remain, specifically: (i) the number of cysts expected in edible tissues of infected animals (ii) quantification of bradyzoites per cyst, (iii) dose-response parameters in humans and (iv) processing and consumption habits at home and how these influence the

risk of viable *T. gondii* being present in the final product. Risk assessments conducted so far have dealt with these knowledge gaps by assuming even distribution of bradyzoites in all tissues ignoring that bradyzoites are 'clustered' within cysts and using dose-response from mice models. Future research should focus on generating evidence that fulfil these data gaps. We show that these assumptions critically affect final risk estimates and propose that caution should be taken when interpreting their findings.

v. *Stochastic hypothetical exposure risk assessment*. Finally, a hypothetical model was developed and is proposed as a tool that could provide a realistic estimate of the risk of exposure to *T. gondii* viable cysts to humans as more data become available. Steps where data generated from this project can be used as input parameters and steps in which data are not currently available are clearly identified. Following identification of the most influential variables in the model, it was clear that results from a formal risk assessment will have significant limitations as a source of information for policymakers regarding the importance of the parasite as a food safety issue until information to fill these key data gaps becomes available. Following simulation of the hypothetical model, the number of viable cysts per 100g of infected edible tissue ranged from 1 to 61 when the best and the worst scenarios were simulated, respectively, crucially the broad range on the number of cysts was originated by those variables for which information is currently lacking.

BACKGROUND

Toxoplasmosis is a zoonotic disease with a worldwide distribution caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*). Most warm-blooded animals (including humans) can get infected and act as an intermediate host in the life-cycle. Felines are the definitive host and the only species able to excrete sporulated oocysts in faeces potentially contaminating the environment, soil and crops used for animal feed (Montoya and Liesenfeld 2004, Andreoletti, Budka et al. 2007). Once ingested, sporozoites released from cysts infect numerous tissues and undergo endodyogeny to form tachyzoites or merozoites which have the ability to infect other tissues such as muscles, liver and nerves. Within the tissues, the parasites develop cyst-like clumping of merozoites that are known as bradyzoites. Livestock can then become infected from the ingestion of infective oocysts in the pastures, feed or drinking water (Andreoletti, Budka et al. 2007).

Humans can become infected through different routes: congenital, ingesting viable cysts in undercooked meat and from accidental ingestion of oocysts from direct contact with cat faeces or contaminated soil, water or unwashed raw vegetables. Ingestion of undercooked meat containing viable cysts appears to be an important source of *T. gondii* infections in some European countries and North America. *Toxoplasmosis* has been ranked as the highest disease burden among foodborne pathogens in the Netherlands (Havelaar, Haagsma et al. 2012) and the USA (Batz, Hoffmann et al. 2011). Pork has ranked as the major source of *T. gondii* infection in the USA (Batz, Hoffmann et al. 2011), whilst consumption of undercooked meat has been reported as one of the major risk factors for infection in pregnant women in various European countries (Cook, Gilbert et al. 2000, Flatt and Shetty 2012).

Detection of *T. gondii* cysts during meat inspection is not feasible given the microscopic size of the cysts. In pigs, a fairly good correlation has been reported between seropositive animals and presence of cysts (Dubey, Gamble et al. 2002, Gamble, Dubey et al. 2005, Hill, Chirukandoth et al. 2006). Contrary, in cattle a lack of correlation between presence of *T. gondii* antibodies and tissue cysts has been reported (Opsteegh, Teunis et al. 2011).

A report containing a risk profile on toxoplasma in the food chain was published in September 2012 by The Advisory Committee on the Microbiological Safety of Food (AMCSF 2012). This report highlighted the need of further studies to estimate level of infection in livestock species in the UK and the frequency and infectivity of *T. gondii* cysts in a range edible tissues, so the burden of

foodborne routes to *T. gondi* human infection can be assessed (AMCSF 2012). Similar knowledge gaps were identified by the European Food Safety Authority (EFSA) (EFSA 2015).

The aim of this project was to generate evidence on the frequency and infectivity of *T. gondii* cysts in meat and other edible tissues and the relationship with *T. gondii* sero-prevalence in the main meat-producing animals. This project aimed to fill some of the key knowledge gaps that currently preclude a formal assessment of potential control options to mitigate the risk of foodborne exposure to *T gondii*. To achieve this overall goal the Food Standards Agency (FSA) collaborated with registered organisations under article 36 of Regulation (EC) 178/2002 in a consortium led by the Dutch National Institute of Public Health and the Environment to investigate the "*Relationship between sero-prevalence in the main livestock species and presence of Toxoplasma gondii in meat*". In the UK, FSA funded studies to be carried out by the Royal Veterinary College (RVC) in collaboration with Moredun Research Institute to conduct observational and experimental studies in cattle and pigs in order to:

i. Assess the relationship between indirect (serology) and direct (bioassay, qPCR, MC-PCR) detection methods for the presence and levels of infective cysts in meat and other edible tissues in cattle.
ii. Evaluate the anatomical distribution of cysts in meat and edible tissues in cattle.
iii. Assess the level of exposure to *T. gondii* in cattle and pigs in the UK and identify farm-level risk factors.

This report summarises the work carried out during the project, including observational and experimental studies conducted as part of the EFSA consortium, as well as further analysis on the cross sectional study in pigs, an abattoir survey of cattle slaughtered in the UK and a hypothetical exposure assessment developed independently of the EFSA project.

LITERATURE REVIEWS

Aim

The aim of the of the extensive literature reviews was to review available data on *T. gondii* in meat of the main livestock species and specifically to provide information on:

- The anatomical distribution of the cysts in meat and other edible tissues.
- The performance of available methods for detecting the presence and infectivity of *T. gondii* cysts.
- The relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues
- On-farm risk factors for *T. gondii* infection in pigs, cattle, small ruminants, poultry and horses.

The methods and results presented in this report are extracted from the report "Relationship between seroprevalence in the main livestock species and presence of Toxoplasma gondii in meat (GP/EFSA/BIOHAZ/2013/01): An extensive literature review" (Opsteegh, Maas et al. 2016)

Methods

Four extensive literature reviews were carried out to compile available data on (i) anatomical distribution of the cysts in meat and other edible tissues; (ii) available methods for detecting the presence and infectivity of *T. gondii* cysts; (iii) the relationship between sero-prevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues and (iv) on-farm risk factors for *T. gondii* infection in the main livestock species.

A detailed description of the search strategy and protocols used is provided by Opsteegh et al (2016). Briefly, one predefined protocol and a data extraction form was developed for each review. The four reviews followed the systematic review approach based on Cochrane guidelines and EFSA guidance including four main steps: identification, screening, data extraction and quality assessment.

Contribution from the Royal Veterinary College and Moredun Research Institute team members were mainly during the protocols development and data extraction stages.

Results

A total of 1651 papers were identified in the first stage, from which 537 were considered non-relevant based on tittle. Following abstract screening (n=1114), 362 papers were excluded and full

text of 752 papers were screened and scored - 342 met the selection criteria and data from these papers was extracted. Some papers met the criteria for more than one of the four reviews.

Anatomical distribution of the cysts in meat and other edible tissues

A total of 88 papers were included in this review. The number of publications and predilection sites identified varied by species. A summed score was used to take into account the ranking of the tissues within each publication and the fraction of studies in which the tissue tested positive. Brain and heart ranked in the top 5 predilection tissues in pigs, sheep, goats, chickens, turkeys and horses. In cattle, however, predilection tissues were different from those identified in the other species with diaphragm ('skirt steak'), lymph nodes and thigh muscle being the top tissues identified. In addition the scores in cattle were lower than in other species (Table 1).

Table 1 Predilection sites for T. gondii in pigs, cattle, small ruminants, poultry and horses

Species	Top 5 tissues	Summed score (W) Range for Top 5	Number of records (studies)
Pigs	brain, heart, tongue, diaphragm, chorio-retinal coat	1.44-0.79	30 (37)
Cattle	diaphragm, unspecified lymph nodes, thigh muscle, small intestine, liver	0.73-0.62	10 (19)
Sheep	brain, heart, skeletal muscle, thorax muscles/ribs, diaphragm	1.30-0.77	12 (17)
Goats	kidneys, brain, heart, liver, skeletal muscle	1.51-1.41	9 (10)
Chickens	heart, brain, ovary duct, ovaries, ventriculus	1.47-0.80	19 (21)
Turkeys	heart, brain, limb muscle, liver, thigh muscle	1.50-1.09	5 (5)
Horses	heart, tongue, small intestine, brain, spinal cord	1.53-0.99	3 (3)

From: Opsteegh, M., et. al, 2016. Relationship between seroprevalence in the main livestock species and presence of T. gondii in meat (GP/EFSA/BIOHAZ/2013/01). An extensive literature review.

Available methods for detecting the presence and infectivity of T. gondii cysts

In total 281 papers were identified reporting results with from at least one method. Mouse bioassay and PCR were the most commonly used methods for direct detection of *T. gondii* in the main livestock species.

After comparing the number of positive samples detected by each method in the studies identified, cat bioassay was the test detecting highest number of positives in more studies, followed by mouse bioassay and PCR (Table 2). The size of the sample was identified as an important factor on the number of positive samples detected suggesting the distribution of infective cysts is uneven and therefore, the larger the sample the most likely is for the parasite be present in the sample tested.

Table 2 Summary of results from test performance comparison

	Number of comparisons	More positives than the method compared with	Less positives that the method compared with
Cat Bioassay	16	14	2
Mouse Bioassay	43	28	15
LAMP	6	4	2
In vitro isolation	2	2	0
PCR	37	19	18
Arg-ELISA	4	1	3
Microscopy with IHC/IFT staining	10	3	7
Non-specific microscopy	28	2	26

Adapted from: Opsteegh, M., et. al, 2016. Relationship between seroprevalence in the main livestock species and presence of T. gondii in meat (GP/EFSA/BIOHAZ/2013/01). An extensive literature review.

Most PCR-based methods performed similarly to mouse bioassay detecting positive samples; however contrary to bioassay, PCR methods do not demonstrate the infectivity of detected parasite.

Studies in which samples were spiked with tachyzoites, bradyzoites or tissue cysts prior to DNA isolation were limited and there were no studies that directly compare different types of direct detection methods (e.g. PCR in comparison to mouse or cat bioassay) using spike samples with a quantified amount of bradyzoites, e.g. by qPCR).

Relationship between seroprevalence in the main livestock species and presence and infectivity of T. gondii cysts in meat and other edible tissues

For this review studies were collected separately by animal species. Publications reporting more than one direct or indirect detection method were separated into different entries.

The number of studies identified varied across species: 13 for pigs (17 entries); 3 for cattle (4 entries); 14 for small ruminants (17 entries); 42 for poultry (76 entries) and 2 for horses (Table 3). Overall a fair to moderate concordance was found in pigs, small ruminants and chickens between detection of antibodies to *T. gondii* and direct detection of the parasite (Table 3). Contrary, data available on cattle and horses suggest a lack of concordance between serology and presence of *T. gondii* in these species, with a low recovery rate of the parasite in seropositive animals and similar rates of direct detection of the parasite in both seronegative and seropositive animals (Table 3).

Table 3 Overall percentage of detection of T. gondii (by cat bioassay, mouse bioassay or PCR in seropositive and seronegative animals by livestock species

Species	Detection in seropositives		Detection in seronegatives		Kappa-value[3] (95 CI)	Concordance	Papers (Entries)
	Overall[1] (95% CI)	Range[2]	Overall[1] (n; 95% CI)	Range[2]			
Pigs	58.8% (54.8-62.8)	8-100%	4.9% (650; 3.3-6.6)	0-75%	0.55 (0.49-0.60)	moderate	13 (18)
Cattle	3.6% (0.14-7.1)	0-10%	2.4% (457; 1.0-3.8)	2-3%	0.02 (<0-0.07)	no to poor	3 (4)
Sheep	39.4% (36.4-42.5)	5-100%	1.8% (0.98-2.7)	0-4%	0.37 (0.33-0.40)	fair	14 (18)
Goats	34.9% (27.3-42.4)	0-72%	2.0% (0.00-5.9)	0-2%	0.20 (0.11-0.28)	poor to fair	4 (4)
Poultry	53.4% (51.0-55.8)	0-100%	1.8-17.4% (1.3-19.0)	0-25%	0.37-0.54 (0.34-0.57)	fair to moderate	42 (76)
Horses	8.8-13.8% (2.6-21.3)	8-9%	2.4-32.0% (1.1-36.0)	3%	<0-0.162(<0-0.23)	no to poor	2 (3)

[1]*Overall percentage of direct detection: the total number of sero-positive (negative) animals per species was used as denominator to calculate the overall % of detection by direct methods (nominator). The total number of sero-positive (negative) animals was obtained by adding up the number of seropositive (negative) animals used in each study (entries). The categorisation into (sero)positive and (sero)negative by direct and indirect detection methods was obtained from each reference used (entries).*

[2]*The range describes the lowest and highest percentage of direct detection obtained from an individual entry (only entries with individually tested animals are considered).*

[3]*Kappa-values were calculated per species based on the direct detection results for seropositives and seronegatives from all entries combined.*

***From:** Opsteegh, M., et. al, 2016. Relationship between seroprevalence in the main livestock species and presence of T. gondii in meat (GP/EFSA/BIOHAZ/2013/01). An extensive literature review.*

Relationship between the on-farm risk factors and T. gondii infection

For this review only publications in which animal husbandry conditions were considered compatible with European husbandry conditions were considered. Publication with more than one specie were split into different entries considering each specie separately. A number of studies provided information on various potential risk and protective factors for *T. gondii* infections in pigs (n=32) and small ruminants (n=32). In other livestock species (cattle, equids and poultry) there were almost no studies available - 3 studies on cattle, 4 studies on equids (including horses, ponies and mules) and 3 studies on chickens.

A summary of the main risk factors identified in the different livestock species is presented in table 4. Overall presence of definitive host, presence of rodents and faecal contamination increased the risk of animals being *T. gondii* positive in all studies. Level of confinement, water source and size of the herd had contradictory results across studies and no clear pattern arose.

Table 4 Risk factors identified for T. gondii infection in main livestock species

Variables related to:	Pigs (n=32)	Cattle (n=3)	Small Ruminants (n=32)	Chickens (n=3)	Equids (n=4)
Definitive host	√	√	√		
Faecal fodder contamination	√		√		
Rodents	√		√		
Biosecurity	√		√		
Housing materials	√		√		
Water provided to animals	√	√	√		
Level of confinement	√	√	√	√	
Size of the herd/ Density	√	√	√		
Specialization	√				√
Purpose of the animal	√		√	√	√
Breed				√	
Climate related	√		√		
Season related	√				

Adapted from: Opsteegh, M., et. al, 2016. Relationship between seroprevalence in the main livestock species and presence of T. gondii in meat (GP/EFSA/BIOHAZ/2013/01). An extensive literature review.

CATTLE STUDIES

Correlation between presence of antibodies and direct detection of *T. gondii* in cattle

Aim

The aim of this study was to assess the correlation between indirect and direct detection of *T. gondii* in cattle (i.e. between the presence of anti *T. gondii* antibodies by modified agglutination test (MAT), mouse bioassay and PCR-based detection on tissue digests and magnetic capture qPCR (MC-PCR)) in a slaughterhouse-based study in four countries: The Netherlands (NL), United Kingdom (UK), Romania (RO), and Italy (IT).

The methods and results presented in this report are extracted from the report "Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01): Final report" (Opsteegh, Schares et al. 2016).

Material and Methods

One hundred animals were sampled in NL, RO and Italy (50 calves and 50 adults) and 102 animals were sampled in the UK. Given that slaughtering of calves in the UK is very uncommon the sampling target was set at 16 calves and 86 adult cattle samples.

Sample collection was performed at slaughter. Cattle were coded with a unique ID. A minimum of 4 ml of blood was collected in a 9 ml serum tube at bleeding or from the heart during evisceration. Based on results from the extended literature review diaphragm and liver were chosen as the tissues to be sampled. A minimum of 200 g of the muscular part of the diaphragm and 400 g of liver was collected in separate sealed bags. Sampling was limited to one animal per farm. Age, sex and breed of each sampled animal were registered using a standardised recording sheet (Annex 1). Samples were kept and transported on ice. The liver was processed for mouse bioassay the day after sample collection. Diaphragm samples were stored at -20°C and, if selected, sent to RIVM for MC-PCR testing.

Serology:

All cattle sera were sent to ANSES – USC EpiToxo in Reims and tested by modified agglutination test (MAT) to detect anti-*T. gondii* antibodies.

Mouse bioassay:

In the UK cattle were tested by mouse bioassay of the liver in Moredun Research Institute. Trypsin digestion of liver and inoculation in two mice per digest was performed. The development of antibodies against *T. gondii* in mice was determined by serology at post mortem (day 42). Samples were tested by ID.Vet ELISA (ID Screen® toxoplasmosis indirect multi-species) and, if necessary, sent to Reims for confirmation by MAT. DNA was isolated from mouse brain homogenates and tested by qPCR targeting a 529bp Repeated Element (RE). For a qPCR reaction to be considered positive all negative or blank controls in the PCR reaction had to be negative, the Cq-value had to be <40 and the shape of the amplification curve had to be 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 (162bp) in gel electrophoresis. Conventional PCR was considered positive when an amplicon of the correct size (529 bp) was identified by gel electrophoresis. A mouse bioassay was considered positive if at least one mouse was positive in serology or PCR.

PCR on liver digest:

DNA was isolated from liver digests using the Nucleospin kit (Machery-Nagel). Samples were subsequently tested by 529bp RE qPCR. This was performed in Moredun Research Institute. PCR was considered positive using the criteria described for PCR on mouse brains.

MC-PCR:

MC-PCR was performed at RIVM on the frozen edible tissue samples for all cattle positive in the mouse-bioassay, PCR on the liver digest or serology. In addition, the edible tissues of 22 cattle negative in bioassay and PCR on the digest, but irrespective of their serological result, were tested by MC-PCR. PCR was considered positive using the criteria described for PCR on mouse brains.

Data analysis:

The agreement 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.

Results

In total 402 animals were sampled (102 animals in the UK). Sixteen calves (between 0 and 2 months) were sampled in a calves' slaughterhouse. One animal sampled in an abattoir for adult cattle was 12 months old and therefore considered calf. Out of the 85 adult cattle sampled, 37 (43.5%) were females between 18 and 90 months and 48 (56.5%) were males between 18 and 38 months.

Detection of antibodies against T. gondii in cattle:

Antibodies were detected by MAT in 12 out 102 cattle tested in the UK. Titres were low, and the maximum titre was 1:200 for one 25 months old cow (Table 5).

Table 5 Modified agglutination test (MAT) titres and classification for cattle from the UK (cut-off value ≥1:6).

Titres	Number of animals			
	UK	IT	RO	NL
1:6	1	1	14	6
1:10	3	1	1	6
1:25	4	2	6	1
1:50	3	0	2	5
1:100	0	0	2	1
1:200	1	0	0	0
Negative	*90*	*96*	*75*	*81*

Adapted from: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Direct detection of T. gondii in cattle in the UK

There was no presence of *T. gondii* demonstrated in the tissues of 102 cattle from the UK.

Mouse bioassay: For sixteen calves all mice inoculated with liver digest died within 24 hours, therefore no mouse bioassay results are available. These calves were younger (0-2 months) than calves sampled elsewhere and toxic effects are suspected.

PCR on liver digest: None of the liver digests tested positive by qPCR.

MC-PCR on diaphragm: Magnetic capture of the 529 bp RE of *T. gondii* was performed on the diaphragms of 34 cattle (12 positive to serology plus an extra 22 negative to Bioassay and serology). All the diaphragm samples tested were negative to MC-PCR (Table 6).

Table 6 Detection of T. gondii in cattle tissues from the UK

Titres	Number of animals			
	UK	IT	RO	NL
Mouse bioassay	0/85	4/100	0/100	2/100
PCR digest	0/102	0/100	0/100	0/100
MC-PCR	0/35	0/30	7/44	0/43

Adapted from: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Concordance between presence of antibodies and detection of T. gondii in tissues

Combining the results for all countries, there was a lack of concordance between presence of antibodies as detected by MAT and detection of *T. gondii* using direct detection methods. For the concordance between MAT and mouse bioassay the kappa-value was estimated at 0.033 (95% CI: <0-0.21) and for MAT and MC-PCR at 0.01 (95% CI: <0-0.13). No positives were detected by PCR on digest, resulting in a kappa-value of 0.0.

Experimental infection with *T. gondii* in calves

Aim

The aim of the study was to determine the dissemination of *T. gondii* tissue cysts to different tissues, organs and cuts of meat intended for the food chain in cattle following infection with *T. gondii* oocysts.

The methods and results presented in this report are extracted from the report "Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01): Final report" (Opsteegh, Schares et al. 2016).

Material and Methods

Since a low prevalence of infective tissue cysts was expected in cattle, it was not considered feasible to study the anatomical distribution of tissue cysts in many different tissues in naturally infected cattle in the slaughterhouse study.

To establish a *T. gondii* infection in cattle, six Holstein Friesians calves (*Bos taurus*), aged 6 weeks, which were seronegative by *T. gondii* ELISA (ID.Vet, Montpellier, France) and MAT at day 0, were each orally infected with $1x10^6$ *T. gondii* oocysts of the M4 strain (day 0). All calves originated from a local farm and were kept at Moredun Research Institute one week prior to inoculation. Calves were housed together and after weaning were fed a commercial calf feed with water available *ad libitum*.

Sampling and measurements

Rectal temperatures of all calves were monitored daily for 18 days post infection. Throughout the experiment blood sampling was carried out weekly (days 0 to 42) by jugular venepuncture into 10 ml vacutainer serum tubes. Blood was left to clot overnight at 4°C then centrifuged at 200*g*, serum transferred to sterile 1.5 ml tubes and stored at -20°C. All calves were euthanized six weeks post infection (day 42) by captive bolt stunning and exsanguination. *Post mortem* examination and collection of tissue samples (brain, heart, diaphragm, masseter, tongue, liver, psoas major (fillet), longisimmus dorsi (sirloin), left triceps femoralis (forelimb) and left semitendinosus (hindlimb), was carried out immediately. Two pools per tissue were prepared, with three calves per pool tested by bioassay and MC-PCR.

All animal procedures complied with the Animals (Scientific Procedures) Act 1986 and were approved by the Moredun Research Institute ethics committee.

Mouse Bioassay

Forty Swiss Webster mice were used for the bioassay of calf tissues. Mice were monitored twice daily with food and water supplied *ad libitum*. Mice were divided into ten separate groups with 2 mice being inoculated with a pool of tissue from 2-3 calves. A total of 100 g of each tissue pool was made from 3 calves (33.3 g per tissue per calf) and included; brain, heart, diaphragm, masseter, tongue, liver, psoas major (fillet), longissimus dorsi (sirloin), left triceps femoralis (forelimb) and left semitendinosus (hindlimb).

DNA extraction from mouse brain and tissue digest

DNA extraction from individual mouse brains and tissue digests were carried out

Calf Serology (MAT)

Calf serum samples were collected weekly throughout the experiment and were tested for *T. gondii* antibodies using the modified agglutination test (MAT).

Mouse Serology

All mouse sera which were collected at the end of the experiment, or at euthanasia due to signs of *T. gondii* infection, were tested by ELISA for *T. gondii* IgG (ID.Vet, Montpellier, France), as described in the manufacturer's instructions. Plates analysed at 450nm using an ELISA microplate reader (MRXII, Thermo Labsystems, UK). An ELISA was valid if the mean value of the positive control OD (OD_{pc}) was greater than 0.350 ($OD_{pc} > 0.350$), and if the ratio of the mean OD values for the positive and negative controls (OD_{pc} and OD_{nc}), were greater than 3.5 ($OD_{pc}/OD_{nc} > 3.5$). Each sample was tested in duplicate.

The OD-readings for the samples were used to calculate percent seropositivity (SP) as described by the manufacturer. A sample with an SP value of 50% or higher was considered positive, a negative result was an SP of 40% or less, and, if the SP was between 40% - 50%, the result was classed as doubtful.

MC-PCR

100g tissue pools were prepared as described for mouse bioassay and sequence-specific DNA isolation using magnetic capture. Remaining tissue samples of liver, heart, diaphragm, triceps femoralis, semitendinosus and masseter were tested individually.

T. gondii 529bp RE quantitative PCR

DNA extracted from mouse brains, tissue digests and DNA samples isolated by magnetic capture were tested by qPCR.

Results

Following oral infection with $1x10^6$ *T. gondii* oocysts, the rectal temperature of all calves began to increase on day 3 and peaked on day 6 (maximum temperature recorded = 41^oC, calf 115). The rectal temperature of one calf consistently remained above 39.3°C from day 13 to 18. This calf was suffering from pneumonia (unrelated to the *T. gondii*) infection and died on day 40.

T. gondii serology in calves.

An initial immune response in calves was observed by day 14, with all animals seropositive for *T. gondii* by MAT on day 21 of the experiment.

Mouse bioassay, digest qPCR and MC-PCR of calf tissues.

Pool 1 consisted of only two animals (112 and 120) as one calf (109) died before the end of the experiment (this death was not due to *T. gondii*). For pool 1 calves, two mice (inoculated with tongue (45-1) and brain (46-1) were culled on days 8 and 6 respectively due to signs of *T. gondii*, which included a starry stiff coat and a hunched appearance. However, parasite DNA could not be detected by qPCR in the brains of these two mice or any of the other mice used for calf tissues from pool 1. In addition all pool 1 tissues tested negative by MC-PCR on the tissue pools. Only one tissue group (triceps femoralis) from the inocula in pool 1 gave a positive reaction in qPCR, but at a very low concentration (Cq 37.20), of which the duplicate sample was negative. This qPCR product was examined by gel electrophoresis and a weak band of the correct size was observed.

For pool 2 calves (115, 111 and 118), mice inoculated with semitendinosus muscle were positive by ELISA and qPCR on mouse brain. Six mice from pool two were culled early (day 12) due to signs of *T. gondii.* These mice had been inoculated with tissue homogenate from tongue, brain and diaphragm and all six tested positive by qPCR. In addition, five out of six of these mice showed a very weak ELISA result (Table 7). An additional eight mice from pool 2 (inoculated with digests from liver, tongue, heart, triceps and psoas major) were qPCR positive in brain, but negative in ELISA. Mouse bioassay results correlate well with MC-PCR results for calf tissues; the only exceptions are a positive result in mouse bioassay for brain of pool 2 and a positive MC-PCR result for masseter in MC-PCR.

MC-PCR of the different tissue pools resulted in Cq-values varying from 32.34 to 35.54. Only three tissues from pool 2 (tongue, diaphragm and semitendinosus) tested positive by qPCR on the inocula.

There were eight cases where mouse serology did not correlate with the positive detection of *T. gondii* parasite DNA from the mouse brain. These eight sera were further tested using the p30 (Tg-SAG1) immunoblot at Friedrich-Loeffler-Institut, and an additional three samples were identified as positive.

Individual testing of the remaining heart, liver, diaphragm, triceps femoralis, semitendinosus and masseter samples by MC-PCR resulted in weak positive reactions for the diaphragm of calf 109 and the heart of calf 112, and positive reactions for triceps femoralis, semitendinosus and masseter of calf 118. Not enough material of other tissues remained for several calves, therefore these tissues were not tested individually.

Table 7 Detection of T. gondii from pooled calf tissues by mouse bioassay, qPCR on tissue digest and MC-PCR

Tissue	Mouse 1			Mouse 2			Inocula	Calf Tissues	Calf Pool
	Cull date	ELISA (%SP)	qPCR (cq)	Cull date	ELISA (%SP)	qPCR	qPCR (cq)	MC-qPCR (cq)	
Liver	42	neg	neg	42	neg	neg	neg	neg	Pool 1 x 2 animals (112 &120)
Masseter	42	neg	neg	42	neg	neg	neg	neg	
Tongue	8	No sample	neg	42	neg	neg	neg	neg	
Heart	42	neg	neg	42	neg	neg	neg	neg	
Brain	6	No sample	neg	3	neg	neg	neg	neg	
Diaphragm	42	neg	neg	42	neg	neg	neg	neg	
Tricep femoralis	42	neg	neg	42	neg	neg	37.20[#]	neg	
semitendinosus	42	neg	neg	42	neg	neg	neg	neg	
Psoas major	42	neg	neg	42	neg	neg	neg	neg	
Longissimus dorsi	42	neg	neg	42	neg	neg	neg	neg	
Liver	42	neg	33.79	42	neg	33.83	neg	34.40	Pool 1 x3 animals (115, 111 & 118)
Masseter	42	neg	Neg	42	neg	neg	neg	35.54	
Tongue	12	24	19.66	12	neg	19.04	33.37	32.34	
Heart	42	neg	34.01	12	neg	33.53	neg	32.84	
Brain	12	12	21.56	42	13	21.70	neg	neg	
Diaphragm	12	8	23.09	42	16	21.47	29.04	34.16	
Tricep femoralis	42	neg	33.60	42	neg	neg	neg	34.63	
semitendinosus	42	285*	18.43	42	230*	19.87	33.92	32.54	
Psoas major	42	neg	18.13	42	neg	19.90	neg	34.09	
Longissimus dorsi	42	neg	neg	42	neg	neg	neg	neg	

Notes on tissue amounts - 33.3g of tissue per animal for MC-PCR; mouse bioassay, pool 1 = 66.6g total (as one calf died prior to the end of the experiment), pool 2 = 100g total.

*Between d10 - 14 mice showed signs of *T. gondii.*

[#]Repeated twice and on both occasions one duplicate was negative.

Adapted from: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Level of *T. gondii* infection in cattle in the UK.

Aim

The aim of this study, which was carried out independently of the EFSA consortium, was to assess the level of infection to *T. gondii* in cattle slaughtered in the UK for human consumption.

Material and Methods

A slaughterhouse based study was conducted in the UK between October 2015 and January 2016. Slaughterhouses were recruited by sending a letter to Official Veterinarians (OVs) in England and Scotland explaining the aim and relevance of the study and asking them if they would be interested in participating. Each slaughterhouse that agreed to take part was visited between two and three times by two team members. On arrival at the slaughterhouse intake sheets were checked along with the OV to verify the number of farms sending animals to slaughter on the day. In addition, the system used in the slaughterhouse for traceability was discussed with the OV.

Sample and data collection

One animal per farm was selected for sampling considering the last farm where the animal stayed. 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 last farm. The first animal of the batch was sampled, if the first animal of the batch was missed, the second animal was sampled.

A minimum of ~150 g of diaphragm muscle was collected at post mortem from the selected animals. Diaphragm samples were placed in polythene bags labelled with a unique ID. In addition, the ID and ear tag numbers from animals sampled were recorded in a standardised recording sheet (Annex 2). Samples were kept and transported on ice and stored at -20°C until ready for use.

Ear tags numbers were used to obtain movement history, age, sex and breed of each animal sampled using the British Cattle Movement System.

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

Laboratory analysis

Samples were prepared for processing and sequence specific magnetic capture as described by Opsteegh and colleagues (Opsteegh, Langelaar et al. 2010). For analysis of DNA obtained after magnetic capture , 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) and 10 µl of template DNA. Cycling conditions were created according to the manufacturer's recommendations for optimized cycling conditions for this mix. This comprised of a polymerase activation and DNA denaturation step at 95°C for two minutes followed by 45 cycles of denaturation at 95°C for 15 seconds and extension at 60°C for 30 seconds. Samples were then cooled to 10°C for five minutes. 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) 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.

Data analysis

Laboratory results and animal details were combined in a relational database in Microsoft Access 2013. Descriptive statistics were obtained at the animal and farm level for all animals tested using R 3.0 (R Development Core Team, 2015). The geographic distribution of farms where sampled animals had stayed was aggregated at regional level using Arc GIS 10.2.2.

Results

Samples were collected from 10 slaughterhouses, nine located across England (1 in the South West, 2 in the South East, 2 in the East Midlands, 1 in the West Midlands, 1 in Yorkshire and Humber and 2 in the North West) and one in Scotland.

Diaphragm samples from 305 animals were collected and tested using magnetic capture and qPCR; 127 (41.6%) were female and 178 (58.4%) were male. The median age of cattle sampled was 765.5 days, with females, on average, being older than males (Table 8). Samples collected represented 34 different breeds or cross breeds (Table 8).

Table 8 Gender and breed of cattle sampled to be tested for T. gondii

Gender	Median age (min – max)
Female (N=127)	798 (346-4482)
Males (N=178)	709 (301-1199)
Breed	**Number of animals (%)**
Limousin cross	54 (17.7%)
Hereford cross	35 (11.5%)
Holstein Friesian	35 (11.5%)
Aberdeen Angus cross	33 (10.8%)
British Blue cross	33 (10.8%)
Charolais cross	19 (6.2%)
Limousin	14 (4.6%)
Simmental cross	13 (4.2%)
British Friesian	10 (3.3%)
Aberdeen Angus	9 (2.9%)
Blonde d'Aquitaine cross	9 (2.9%)
Charolais	7 (2.3%)
Dexter	5 (1.6%)
Holstein Friesian cross	4 (1.3%)
British Blue	2 (0.7%)
British Friesian cross	2 (0.7%)
Hereford	2 (0.7%)
South Devon cross	2 (0.7%)
Sussex cross	2 (0.7%)
Other *	15 (4.9%)

* Includes: Ayrshire, Bazadaise cross, Beef shorthorn, Blonde d'Aquitaine, Holstein, Red Poll cross, Salers, Shorthorn, Shorthorn cross, Simmental, South Devon, Stabiliser, Swedish Red and white cross, Welsh black cross and Welsh White

The number of sites an animal stayed before slaughter ranged from 1 to 15 (median 3). Overall the 305 animals sampled covered 614 different farms and 40 livestock markets across the country. The number of farms and livestock markets, aggregated by region, is displayed in Table 9.

Table 9 Number and percentage of livestock markets and farms, per region, where sampled cattle 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	1 (2.5%)	52 (8.5%)
•Humber	2 (5%)	45 (7.3%)
•Northwest	-	8 (1.3%)
•Northeast		
Scotland	6 (15%)	44 (7.2%)
Wales	6 (15%)	64(10.4%)
Total	40	614

Five (1.6%) samples were deemed positive following magnetic capture and qPCR. All positive samples came from male cattle and age at slaughter ranged between 467 and 952 days (Table 10). The five positive animals were slaughtered in four different slaughterhouses. None of the five positive animals stayed in the same site (farm or livestock market) before being slaughtered (Table 10). Cq values obtained from qPCR ranged from 17.03 to 44.03. The animal with lowest consistent Cq values (C278) came from Scotland. Analysis of sample C006 was repeated following equivocal results from the first assay (one well negative, one positive).

Table 10 Characteristics of cattle that tested positive to Toxoplasma gondii using qPCR

Animal ID	Sex	Breed	Age (days)	Number of sites stayed	Region(s)	qPCR(cq)
C006	Male	Limousin	551	5 (3 farms; 2 livestock markets)	Southeast and East Midlands	35.08; 17.03
C172	Male	Charolais	952	3 (3 farms)	Northeast and Yorkshire and the Humber	20.35; 20.30
C201	Male	Simmental cross	774	3 (2 farms; 1 livestock market)	Southwest	38.43; 44.03
C278	Male	Limousin	467	3 (2 farms; 1 livestock market)	Scotland	19.66; 19.53
C289	Male	Charolais cross	601	3 (2 farms; 1 livestock market)	Scotland	19.82; 18.90

*Animal C006 tested -/35.8 during the first assay, recording 17.03 when re-tested using the same 100g.

PIG STUDY

Level of exposure and risk factors for *T. gondii* infection in pigs in the UK

Aim

The primary objective of this study was to assess the level exposure to *T. gondii* in pigs raised in selected commercial farms in England and to identify factors associated with a higher risk of exposure. A secondary objective was to compare the performance of a commercially available ELISA with modified agglutination test (MAT) using sera from commercial pigs. The study was partially carried out as part of the EFSA consortium and elements of the methods and results sections presented in this report have been extracted from *"Experimental studies on Toxoplasma gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01): Final report". Extra testing and analysis was carried out independent to the EFSA consortium* (Opsteegh, 2016).

Material and Methods

Study design

A cross sectional study was conducted in England between January and July 2015 with the pig batch as the unit of interest. Batches of pigs were recruited in slaughterhouses. Firstly, a note explaining the aim and relevance of the study was published in the British Pig Executive (BPEX) newsletter in December 2014 (Annex 3). As a result, five commercial slaughterhouses volunteered to take part in the study; they varied in size and throughput from 5 to 2000 pigs processed a day. Farmers regularly sending pigs to these slaughterhouses were contacted and invited to take part in the study.

The study received ethical approval from the Royal Veterinary College Ethics and Welfare Committee under the reference URN 2015-1328

Samples and data collection

Batches of pigs sent to the collaborating abattoirs from farmers that agreed to participate were included in the study. From each batch, blood samples were collected from individual pigs during routine slaughter at the point of bleeding (sticking). Nine millimetres of blood were collected from each pig using pre-labelled vacutainer tubes. Up to 25 pigs were sampled per batch. For large batches every third animal was sampled until the required sample of 25 pigs was achieved; whilst for

small batches (less than 25 pigs) all pigs in the batch were sampled. Date of sampling and gender were recorded.

Information on farm characteristics, management practices and biosecurity were gathered using a standardised questionnaire (Annex 4). The questionnaire was either sent by post (with a pre-paid envelope to be posted back) or given directly to farmers when they took the pigs to the slaughterhouse themselves. Farmers had the option to fill in the questionnaire on the same day or take it with them to fill it in later and return via post using the pre-paid envelope provided.

Serology

Blood samples were centrifuged to separate sera from blood cells and sera samples were tested using the Modified agglutination test (MAT) for the detection of *T gondii* specific immunoglobulin (IgG). Testing was performed at French Agency for Food, Environmental and Occupational Health and Safety in Reims, France as previously described by Dubey & Desmonts (Dubey and Desmonts 1987). A sample was considered positive if it had titres ≥1:25 and suspicions if titres were between 1:1 and 1:10.

All positive and suspicions samples by MAT from which sera samples was available plus a subset of randomly-selected negative samples were tested in duplicate by a commercially available enzyme-linked immunosorbent assays (ELISA) (ID Screen® toxoplasmosis indirect multi-species). ELISA's were performed according to manufacturer's instructions at the Royal Veterinary College. The OD-readings for the sample were used to calculate percentage seropositivity (SP) as described by the manufacturer. A sample with an SP value of 50% or higher was considered positive, an SP of 40% or less was a negative result and between 40% and 50% was considered doubtful. Testing was repeated (also in duplicate) for those samples which had contradictory results during the first ELISA test (i.e. one well classified as positive and one negative or doubtful). If after repeating the test results were still contradictory the sample was considered inconclusive.

The agreement between the MAT and ELISA was determined by the Kappa coefficient (κ) excluding inconclusive results. Repeatability between ELISA results was measured using the coefficient of variation (CV). The CV of each sample was calculated for all the replicate values and then averaged across all of the 492 samples.

Data analysis

Questionnaire data and laboratory results were entered into a relational database in Microsoft Access 2013. Data cleaning was done during the preliminary descriptive analysis. Descriptive statistics were obtained (i) at animal level for all pigs sampled (n=2071) and (ii) at farm level for farms which completed the questionnaire (n=73).

Animal level risk factor analysis

The extent to which gender was associated with animal infection was determined using a logistic regression including farm as a random effect. Animals with sera titres ≥1:25 were considered positive and suspicious results were considered negative.

Sero-prevalence estimation

A Bayesian model previously constructed in R (R Core Team, 2015) was used to estimate the farm-level prevalence (Beauvais, W., et al under review). Briefly, the probability of each farm having at least one true positive pig was estimated after taking into account the number of pigs that were tested, how many of them were found to be positive, the imperfect sensitivity and specificity of the test and the uncertainty arising from sampling only a proportion of animals on each farm. For each iteration of the model, based on the probabilities of each farm being positive, we simulated the overall farm-level prevalence. The results for each iteration were combined to create an uncertainty distribution for the true farm-level prevalence. The median value of this uncertainty distribution was taken as the adjusted farm-level prevalence. Sera titres ≥1:25 were considered positive. A sensitivity and specificity of 86% and 95%, respectively were used as inputs (Gamble, Dubey et al. 2005). Model results were used to classify farms as positive or negative using two cut-offs: positive farms those for which the probability of having at least one true positive pig was ≥0.50 (cut-off 1) or those for which the probability was ≥0.10 (cut-off 2).

Identification of risk factors for T. gondii infection at farm level

Putative predictors of exposure to *T. gondii* within a farm were categorised on the basis of answers given in the questionnaire and risk factors previously identified in the literature (Table 11).

Crude associations between predictor variables and farm status were tested by means of Fisher's exact test or Pearson's Chi squared test as appropriate; relative risk was calculated as a measure of strength of association. Collinearity was assessed between all predictor variables for which $p \leq 0.05$ in the univariate analysis and when present ($p<0.1$) only one of the variables was kept for further multivariate analysis. Logistic models were built to assess the relationship between the individual

predictor variables and the outcome, accounting for the potential confounding effect of other variables. Odds ratios were obtained as a measure of effect for the association between predictor and outcome.

Statistical analysis was performed in R 3.0 (R Development Core Team, 2015) using packages epicalc and lme4.

Table 11 Variables considered as putative risk factors for T. gondii infection in commercial pigs. Information collected between January and July 2015 by means of standardised questionnaire (n=73)

Variable description and question asked in the questionnaire	Categories / options provided in the questionnaire	Variable re-grouped for analysis	
FARM CHARACTERISTICS			
Production cycle ***Which of the following describe the production cycle in the farm?***	-Farrow to finish	Complete cycle	
	-Breeding to weaning -Weaning to finishing -Grower to finishing	Part of the cycle	
Source of the farm pigs ***If weaning to finishing or grower to finishing, where did you get the pigs from the last batch sent to the slaughterhouse?***	-From a unit placed in another site but part of the same farm (same owner)	Same owner	
	-From another farm (different owner) -From different farms -Other (please specify)	Another farm(s) different owner	
Farm holdings ***Do you keep pigs in more than one site/holding?***	-Yes -No	Yes No	
Production system ***What is the production system in the farm?***	-All in all out -By farm -By site -By building -By pen	All in all out	
	-Continuous -Other (Please specify)	Continuous	
Outdoor access ***Using the definitions provided below, please complete the table by ticking the box that best describes the way animals are kept in the farm*** ***<u>Indoors</u> is defined as keeping pigs in enclosed buildings (i.e. delimited by solid walls) and pigs are not able to go outside the building.*** ***<u>Outdoors</u> is defined as kept in the field within defined boundaries where they are free to roam and are provided with food, water and shelter.***	Asked per production stage and 3 possible options (keep outdoor all the time, keep indoor all the time and keep part of the time outdoor and part indoor) dry sows lactating sows boar piglets weaners growers finishers → outdoor / indoor / part outdoor part part indoor	Have outdoor access at any production stage Yes No	
Number of animals ***Please fill in the table below indicating the total number of pigs for each production stage at this moment***	Number of pigs hold in each production stage in the farm	Total number of pigs (continuous) 1-220 pigs; >220pigs	
Other livestock species ***Are there other livestock species (apart from pigs) in this site?***	-Yes -No	Yes No	
FOOD AND WATER Food storage ***Where is the animal feed stored? Tick all that apply***	-Open silo -Open storage -Close silo -Close storage -Bags for food -Other (Please specify)	Open storage (Yes/No)	
Type of feeders ***Which types of feeders are used in this site? Tick all that apply***	-None (floor) -Dump feeders	On the floor (Yes/No)	•Off the floor only •Either all on the floor or some on the floor and some off floor
	-Individual feeders -Bowl -Pipeline -Other (Please specify):	Off the floor (Yes/No)	

Variable description and question asked in the questionnaire	Categories / options provided in the questionnaire	Variable re-grouped for analysis
Pigs' drinking water ***Where does the pigs' drinking water come from? Tick all that apply***	-Main supply (community tap water)	Main supply
	-Local canal / stream -Well -Other (Please specify)	Other (local canal/stream, well or bore)
BIOSECURITY Cleaning between batches ***Is it common practice to clean between batches?***	-Yes, it is always cleaned between batches -Yes, most of the times it is cleaned between batches	Yes
	-Rarely -NA (Continuous system)	No
Disinfect between batches ***Is it common practice to disinfect between batches?***	-Yes, it is always cleaned between batches -Yes, most of the times is cleaned between batches	Yes
	-Rarely -NA (Continuous system)	No
Staff ***Are staff designated to work exclusively in certain areas of this site?***	-Yes -No	Yes No
Keep cats ***Do you keep cats in this site?***	-Yes -No	Yes No
Cats no belonging to the farm ***Is it possible that cats not belonging to this site get into the site?***	-Yes -Not sure	Possible
	-No	No
Cats – contact with pigs ***Is it possible that cats come into direct contact with the pigs?*** Cats – contact with pigs' food ***Is it possible that cats come into contact with pigs' food?*** Cats – contact with pigs' drinking water ***Is it possible that cats come into contact with pigs' drinking water?***	-Yes, cats definitely come into direct contact with pigs / pigs' food / pigs' drinking water -Yes, it is very likely that cats come into contact with pigs/ pigs' food / pigs' drinking water -Not sure	Possible
	-No, cats cannot come into contact with pigs/ pig's food / pigs' drinking water	No possible
PREVENTIVE MEDICINE Wormers ***Please complete the table below concerning the routine de-worming used on the farm***	Asked per production stage dry sows lactating sows boar piglets weaners growers finishers product used and frequency	Yes / No

Adapted from: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Results

In total 2071 pigs from 131 farms were sampled; 1101 females, 953 males (gender was not recorded from 17 pigs). Antibodies (IgG) against *T. gondii* were found in 155 pigs (7.5%) using MAT, but only 75 pigs (3.6%) had titres ≥1:25 (Figure 1). Gender was not significantly associated with *T. gondii* infection (p=0.14).

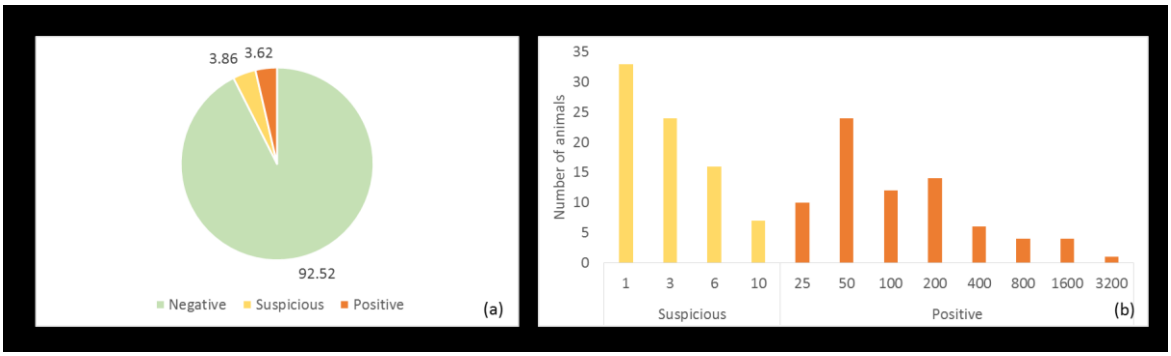

Figure 1 (a) Proportion of negative, suspicious (titre 1:1, 1:3, 1:6 and 1:10) and positive (titre ≥1:25) pigs (n=2017) and (b) number of animals in each titre band. Samples collected between January and July 2015. Results in this figure are not adjusted for the Sensitivity and Specificity of the test. From: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Titres for samples positive by MAT and not by ELISA ranged from 1:25 to 1:400. Three samples were positive to ELISA and negative to MAT and 2 samples classified as suspicious by MAT (titre <1:25) were positive with ELISA (Table 12). The values of the Kappa statistic for the results obtained by MAT and ELISA tests showed moderate agreement: Kappa=0.61 (95% confidence interval 0.52-0.70), with a higher number of samples classified as positive by MAT than by ELISA (Table 12). For repeated samples, the mean CV values for ELISA test were 0.62, therefore there was substantial variation and low precision of the test.

Table 12 MAT titres and ELISA results for pig serum samples (n=492) collected between January and July 2015. Results in this table are not adjusted for the Sensitivity and Specificity of the test

MAT		ELISA		
Status	***Titre***	***Negative***	***Positive***	***Inconclusive***
Negative	**0**	**334 (67.9%)**	**3 (0.61%)**	**3 (0.61%)**
Suspicious	1:1	31	1	0
	1:3	21	0	3
	1:6	13	1	2
	1:10	3	0	4
	Total	**68 (13.8%)**	**2 (0.41%)**	**9 (1.8%)**
Positive	1:25	6	2	2
	1:50	11	8	5
	1:100	5	5	1
	1:200	6	7	1
	1:400	1	5	0
	1:800	0	3	1
	1:1600	0	2	2
	1:3200	0	0	0
	Total	**29 (5.9%)**	**32 (4.9%)**	**12 (2.4%)**

Twenty four farms out of 131 sampled had at least 1 animal positive (apparent prevalence 18.3%). The adjusted prevalence was 11.5% (95% credible interval 8.4%-16.0%); this is a sample estimate and should not be considered as a population estimate.

Seventy three farms (55.7%) returned a completed questionnaire which were located across England: 16 (21.9%) in the Southeast, 12 (16.4%) in the Southwest, 6 (8.2%) in East England, 5 (6.8%) in the East Midlands, 8 (11.0%) in the West Midlands, 18 (24.7%) in Yorkshire and the Humber, 5 (6.8%) in Northwest and 3 (4.1%) in Northeast). Two thirds of them were farrow to finish and 60% had a continuous production system. More than half (56%) kept other livestock in the farm, with sheep and cattle being the most common species kept. The median number of animals was 220 pigs (min 3 - maximum 9756 pigs). In almost half of the farms (48%) pigs had outdoor access for some stage of the production cycle. Twenty seven farms (37%) had cats on the site and 62% considered it was possible for cats not belonging to the site accessing the farm (Table 13).

Farm-level risk factors

From those farms that returned a completed questionnaire (n=73), only 5 farms were deemed positive using a cut off of ≥50% probability of having at least one true positive animal (Figure 2). There was no statistically significant association (P≤0.05) between positive farms and any of the putative risk or protective factors explored (Table 13). However some patterns did arise, not surprisingly the presence of cats (either belonging to the farm or coming from outside) increased the

risk 2.5 fold. The relative risk of a farm being positive was higher for those farms having an open feed storage (RR=5.8), farms in which cats could access pigs' food (RR=2.6), having off floor feeders (RR=3.1) and not cleaning between batches (RR=2) suggesting that not only the presence of cats is important but also the unnoticed feed contamination with cat's faeces containing sporulated oocysts.

Fifteen farms were deemed positive considering a lower cut off: ≥10% probability of having at least one true positive. Three farm characteristics were statistically significant from the univariate analysis; the relative risk of a farm being positive was higher in those farms having outdoor access (RR=3.0; P=0.04), holding up to 200 pigs (RR=3.9; p=0.02) and cats having direct access to feed (RR=2.6; p=0.04) (Table 13). These 3 variables exhibited strong collinearity (p<0.1). Therefore, only cats having direct access to pigs feed was kept in the final model (Table 14).

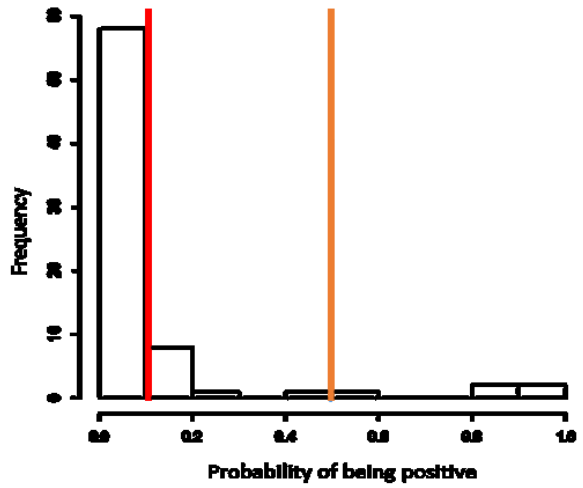

Figure 2 Probability of each farm being positive after adjusting for test sensitivity and specificity and proportion of animals sampled in each batch. Cut-off used to consider farms positive or negative are illustrated with red (≥10%) and yellow (≥50%) lines. Adapted from: Opsteegh, M., et. al, 2016. Experimental studies on T. gondii in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report

Table 13 Distribution and crude association of putative risk factors for T. gondii infection in pigs at slaughter considering two cut off for a positive status (≥50 and 10% probability) (N=73).

Risk factor	≥50 probability of being a positive farm				≥10 probability of being a positive farm			
	No. negative (%)	No. positive (%)	*P*	Relative Risk	No. negative (%)	No. positive (%)	*P*	Relative Risk
Production cycle								
• **Complete cycle**	45 (66.2)	2 (40.0)			37 (63.8)	10 (66.7)		
• **Part of the cycle**	23 (33.8)	3 (60.0)	0.34	2.7	21 (36.2)	5 (33.3)	1	0.9
Source								
• **Same owner**	51 (25.0)	2 (40.0)			15 (25.9)	5 (33.3)		
• **Different owner**	17 (75.0)	3 (60.0)	0.12	4.0	43 (74.1)	10 (66.7)	0.56	0.75
Farm holdings								
• **More than one site**	18 (26.5)	1 (20.0)			40 (69.0)	14 (93.3)		
• **One site**	50 (73.5)	4 (80.0)	1	1.4	18 (31.0)	1 (6.7)	0.10	0.2
Production system								
• **All in all out**	26 (38.8)	3 (60.0)			25 (43.9)	4 (26.7)		
• **Continuous**	41 (61.2)	2 (40.0)	0.39	2.2	32 (56.1)	11 (73.3)	0.26	1.6
Outdoor access (at any production stage)								
• **No**	36 (52.9)	2 (40.0)			34 (58.6)	4 (26.6)		
• **Yes**	32 (47.1)	3 (60.0)	0.67	1.6	24 (41.4)	11 (73.3)	0.04	3.0
Farm size								
• **Large herds (>200 pigs)**	34 (50.0)	3 (60.0)			33 (56.9)	3 (20)		
• **Small herds (1-200 pigs)**	34 (50.0)	2 (40.0)	1	1.2	25 (43.1)	12 (80)	0.02	3.9
Hold other livestock species in the farm								
• **No**	31 (45.6)	1 (20.0)			28 (48.3)	4 (26.7)		
• **Yes**	37 (54.4)	4 (80.0)	0.38	3.1	30 (51.7)	11 (73.3)	0.16	2.2
FOOD AND WATER								
Food storage open								
• **No**	66 (97.0)	4 (20.0)			56 (96.6)	14 (93.3)		
• **Yes**	2 (3.0)	1 (80.0)	0.19	5.8	2 (3.4)	1 (6.7)	0.50	1.7
Type of feeders								
• **On floor (some or all)**	31 (45.6)	1 (20.0)			33 (56.9)	8 (53.3)		
• **Off floor only**	37 (54.4)	4 (80.0)	0.37	3.1	25 (43.1)	7 (46.7)	0.80	1.1
Pigs drinking water: stream well or bore								
• **No**	49 (26.5)	3 (60.0)			39 (67.2)	13 (86.7)		
• **Yes**	19 (73.5)	2 (40.0)	0.62	1.6	19 (32.8)	2 (13.3)	0.20	0.4
BIOSECURITY								
Cleaning between batches								
• **Yes**	28 (41.2)	3 (60.0)			31 (53.4)	11 (73.3)		
• **No**	40 (58.8)	2 (40.0)	0.65	2.0	27 (46.6)	4 (26.7)	0.24	0.5
Disinfect between batches								
• **Yes**	29 (42.6)	3 (60.0)			30 (51.7)	11 (73.3)		
• **No**	39 (57.4)	2 (40.0)	0.65	1.9	28 (48.3)	4 (26.7)	0.16	0.5
Staff working exclusively in certain areas								
• **Yes**	10 (14.7)	2 (40.0)			49 (84.5)	12 (80)		
• **No**	58 (85.3)	3 (60.0)	0.18	3.4	9 (15.5)	3 (20)	0.70	1.3
Keep cats in the farm								
• **No**	44 (64.7)	2 (40.0)			38 (65.5)	8 (53.3)		
• **Yes**	24 (35.3)	3 (60.0)	0.35	2.6	20 (34.5)	7 (46.7)	0.38	1.5
Cats not belonging to the farm get into the site								
• **No**	27 (39.7)	1 (20.0)			25 (43.1)	3 (20)		
• **Possible**	41 (60.3)	4 (80.0)	0.64	2.5	33 (56.9)	12 (80)	0.14	2.5
Cats can get in contact with pigs								
• **No**	29 (42.6)	2 (40.0)			27 (46.6)	4 (26.7)		
• **Possible**	39 (57.4)	3 (60.0)	1	1.1	31 (53.4)	11 (73.3)	0.24	2.0
Cats can get in contact with pigs' food								
• **No**	44 (64.7)	2 (40.0)			40 (69.1)	6 (40)		
• **Possible**	24 (35.3)	3 (60.0)	0.35	2.6	18 (31.0)	9 (60)	0.04	2.6
Cats can get in contact with pigs' drinking water								
• **No**	44 (64.7)	3 (60.0)			44 (64.7)	3 (60)		
• **Possible**	24 (35.3)	2 (40.0)	1	1.2	24 (35.3)	2 (40)	0.11	2.1
PREVENTIVE MEDICINE								
Deworm in at least one production stage								
• **No**	30 (44.1)	4 (80.0)			27 (46.6)	7 (46.7)		
• **Yes**	38 (55.9)	1 (20.0)	0.18	0.2	31 (53.4)	8 (53.3)	0.99	1.0

Table 14 Results of a logistic regression model for the identification of risk factors for the presence of T. gondii considering ≥10% probability cut off for positive status

	Categories	Crude OR (C.I.)	*p*
Cats can get in contact with pigs' food	No	1	
	Possible	3.3 (1.03- 10.77)	0.04

RISK ASSESSMENT

The aim of this component was to develop a hypothetical stochastic model that could be used as the foundation for a formal quantitative RA as more data become available. This component was carried out independently of the EFSA consortium.

Review of previous Risk Assessments for meat-borne toxoplasmosis

To date, only three risk assessments (RA) estimating the relative contribution of different meat products to human *T. gondii* burden have been published. The first was a qualitative RA published in 2008 and aimed at estimating the risk of exposure to *T. gondii* cysts via consumption of ready to eat meats in Australia (Mie, Pointon et al. 2008). Three years later a quantitative RA aiming to quantify the relative contribution of sheep, beef and pork products to human *T. gondii* infection in The Netherlands was published (Opsteegh, Prickaerts et al. 2011). Finally, in 2015, a qualitative RA was published estimating the relative risk of exposure to meat products consumed in the United States, evaluating the effects of meat processing on the survival of *T. gondii* (Guo, Buchaman et al. 2015). All the RAs followed the guidelines provided by the Codex Alimentarius Commission. A brief description of the main assumptions, outputs generated and main data gaps identified in each RA are presented in Table 15.

Although some of the data gaps identified were specific to the country or the products evaluated, all three RAs identified the following critical knowledge gaps: (i) the probability of viable cysts being present in edible tissues given the animal is infected, (ii) the probability distribution describing the number of viable cysts in tissues of infected animals, (iii) the quantification of bradyzoites per cyst, (iv) dose-response parameters in humans and (v) processing and consumption habits at home and how these influence the probability of viable cysts being present in the final product.
These data gaps relate to key inputs in the models. Therefore, numerous assumptions in important parameters were made in all RAs and the validity of the model estimates as measures of the burden of *T. gondii* foodborne transmission on human toxoplasmosis is unknown.

Table 15 Key assumptions, outputs generated and main data gaps identified in each RA previously published.

Reference / Type	Key assumptions				Outputs	Gaps acknowledge in the risk assessment	
	Relationship between serology, infection and presence of cysts	***Relationship between livestock species infection and presence of cysts.***	***Effectiveness of meat processing (i.e. heating, freezing, salting, etc) inactivating viable cysts***	***Amount and type of meat consumed and dose response***		**Specific to the RA**	**Common to the three RAs**
Mie, T., et al. (2008). *Qualitative*	The proportion of infected livestock is inferred from results of serological studies in the country. It is assumed that seropositive animals have viable tissue cysts. Density of cysts is assumed to be different across tissues.	Products containing pigs, sheep and goat meat, as raw ingredient, have higher probability of containing viable cysts than those containing beef (and therefore the risk is higher)	All tissue cysts become non-viable after heat treatment or cooking regardless of the time/temperature combination Salt, in combination with official time and temperature requirements, reduce the risk by one level (e.g. from medium to low) Salt concentration is assumed to be homogeneous distributed throughout the final product in all products. All tissue cyst become non-viable after freezing and the risk of the final product is reduced to minimal risk category.	*Not considered. The assessment only considers risk exposure at retail.*	Ready to eat meats might contain viable cysts and pose a risk to consumers. The likelihood of containing viable cysts mainly depends on the processes the product undergoes. Products undergoing heat treatment had the lower likelihood of containing viable cysts.	Up to date *T. gondii* seroprevalence on livestock species in Australia.	Probability that an animal is infected given that it is seropositive. † Probability of cysts being present in an edible tissue of interest given that the animal is infected. † Density of cysts and number of bradyzoites per cyst in different tissues given that an animal is infected. Effect of different meat processes (e.g. Freezing heating, salting) and duration of the process on cysts / bradyzoites viability. Dose-response relation for *T. gondii* in humans
Opsteegh, M., et al. (2011). *Quantitative*	The probability of infection was inferred from prevalence studies in the country (using serology or a molecular method). It was assumed that the concentration of bradyzoites in skeletal muscle of an infected animal was the same regardless of the species and tissue and equal to the findings from 35 sheep heart samples.	For sheep and pork products, the probability that a portion is infected is assumed equal to the seroprevalence for the species. For beef products, the probability that a portion is infected is assumed equal to the prevalence by PCR in cattle.	Effect of processing (salting, freezing and heating) on the number of bradyzoites was modelled based on inactivation experiments using results from bioassay in mouse. For salting, parameters stated in the packing of each product were used. For freezing, storage was assumed to be evenly distributed on all the portions. It was not assumed that heating duration was always 0.01 min.	The number of portions consumed of different meat products was derived from a survey representative of the Dutch population, assuming homogeneity of meat consumption during the year. The *T. gondii* dose-response relationship in humans was considered to be the same as in mice.	Estimated $2.8x10^{6}$ new infections in the susceptible Dutch population and $2.6x10^{4}$ new infections in susceptible pregnant women 67.6% of the meat-borne infections were attributed to beef; 14% to sheep and 12% to pork	Freezing of meat in or prior to retail Consumer behaviour when cooking meat Temperature / time used when cooking meat at home. Prevalence of different *T. gondii* genotypes.	

Guo, M., et al. (2015). *Qualitative*	The proportion of infected animals is inferred (per species) from results of seroprevalence studies in the country; when no published papers were available expert opinion was used.	When combining meats previously identified with a different risk category, the new meat product kept the higher risk status from the two products.	Freezing reduces the risk to the minimal level regardless of the risk estimated previous to freezing. Injection of enhancing solution, curing, drying, fermentation, irradiation and hydrostatic pressure reduce the risk status by one level. Smoking alone does not reduce the risk status but hot-smoking reduces the risk to the minimum level. Thermal processing reduces the risk to the minimal level if the minimum temperature and time (specific to tissue and species) are met.	*Not considered. The assessment only considers risk exposure at retail.*	Exposure risk of *T. gondii* posed by meat purchased at retail stores in the US is low. Fresh meat, fresh processed meat, raw fermented sausages and meat products that are not hot-air dried pose a higher risk than other meat products.	Probability and magnitude of cross-contamination occurring during slaughter process Survival of *T. gondii* tissue cysts in low water activity environment Relationship between number of bradyzoites and the age of the tissue cysts	

† Knowledge gaps partially filled in by this report.

In conclusion, previous RAs made several strong assumptions regarding key input parameters which were critical in the final risk estimation. The current project has generated evidence in relation to some of these assumptions that would be useful for future risk assessments in different countries and settings. In the UK context specifically, evidence has been generated on the level of *T. gondii* infection in pigs and cattle.

However, some key gaps such as number and distribution of viable cysts in tissues of infected animals, number of bradyzoites per cyst and dose-response parameters in humans still remain. Performing another quantitative risk assessment based on numerous assumptions and high levels of uncertainty would produce outputs with limited practical use for policy makers. Therefore, the development of a well-structured hypothetical model that could be used as the foundation for a formal quantitative RA, as more data become available, was deemed to be of more value.

In the next section a stochastic hypothetical model aimed at producing a probability distribution describing the occurrence of at least one viable cyst in a 100g portion of a hypothetical edible tissue ($N_{cysts\_}p_{Ti}$) is presented. Differently from the only quantitative RA published so far, our approach uses the 'cyst' as the biological unit at which humans are exposed.

Although the use of the number of bradyzoites/g without proceeding to the conversion in the number of cysts/portion is a significant and attractive simplification from a modelling prospective, such approach implies a number of critical assumptions. In fact, as bradyzoites are located in tissue cysts and not distributed homogeneously in the muscular tissue, the cysts are the first biological units which the consumers are exposed to and the consumption of a meat product containing at least one tissue cyst is the first conditional step to become infected. This conceptual difference has proven to be of great impact in the modelling of human exposure, especially when products that are consumed in small portions or composite food matrices such as minced meat are considered (Opsteegh, Prickaerts et al. 2011). In fact, in the first case it is assumed that the amount of bradyzoites ingested is directly dependent on the grams of meat consumed and not on physical presence of the cyst in that area; while in the second case (adopting the bradyzoites/g as measure unit) leads to unrealistic dilution effects when meat from infected and not-infected animals are mixed together.

Following these considerations, our hypothetical approach for the assessment of human exposure to *T. gondii* through consumption of meat product adopts the number of cyst per portion as the biological measure unit. Steps for which data are currently unavailable are highlighted and the implications of this on the final estimate are discussed.

Hypothetical exposure risk assessment

The hypothetical model is structured in four main steps. The flowchart of the model is outlined in figure 3.

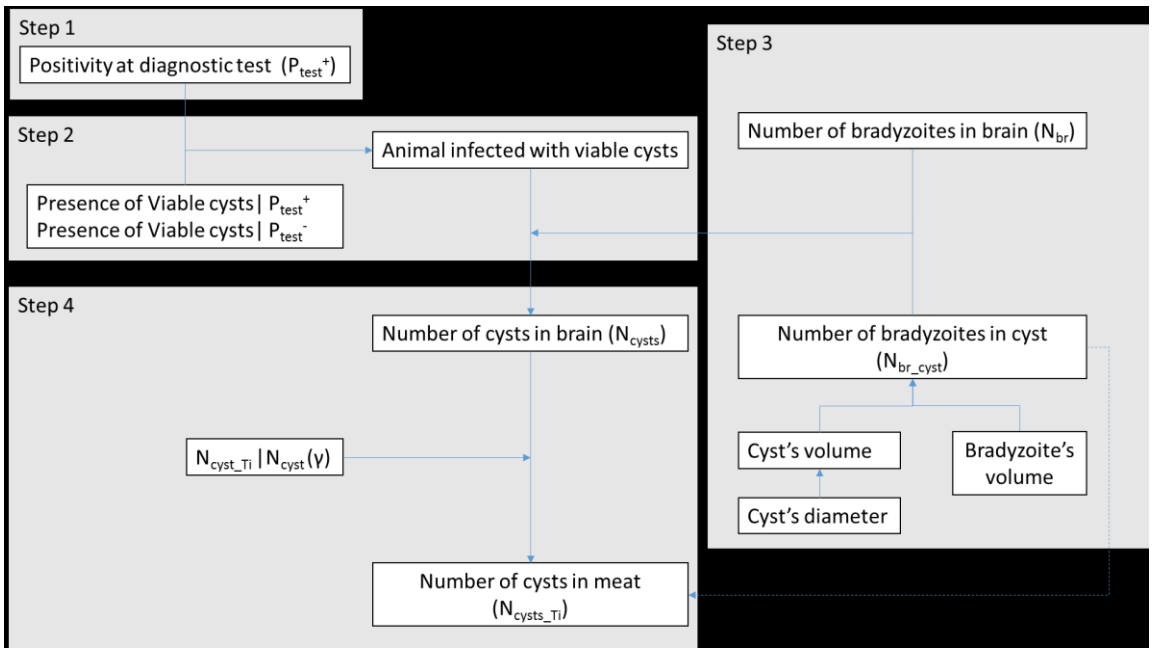

Figure 3 Flowchart of the hypothetical exposure assessment model showing the input and the steps involved

Each step is described below in detail specifying if data generated from the project were used or if a hypothetical distribution was required given the lack of data.

Step 1. True prevalence of animals exposed to T. gondii

This step gives the estimated true prevalence of either seropositive animals or PCR-positive animals in the target population, given the number of animals tested, the number of animals found positive out of those tested and the Sensitivity (*Se*) and Specificity (*Sp*) of the tests used. Therefore this step can be used to obtain true prevalence estimates from serological surveys that detect antibodies in serum or surveys that use PCR for *T. gondii* DNA detection in tissue samples. It should be noted that those tests are not indicative of the actual presence of viable parasites in meat, and the prevalence distribution obtained in this step should be considered as the proportion of animal that are or have been exposed to *T. gondii* in the population. While it is recognized that serology is a suitable means of estimating prevalence in pigs, the low correlation between serological results and infection status in cattle means that direct methods are needed to estimate prevalence in cattle.

The distribution describing the uncertainty surrounding the true prevalence estimates obtained by means of serological (P_{SERA}^{+}) or molecular (P_{DNA}^{+}) testing in the population was obtained from the following epidemiological data: number of animals tested (*n*); number of animals that tested positive (*s*) and Sensitivity (*Se*) and Specificity (*Sp*) of the diagnostic test adopted following a Binomial process and applying the Bayes theorem:

$$f(\theta|X) \propto \pi(\theta) * L(X|\theta) \qquad \text{eq.1}$$

Where π (θ) is the density function of the prior belief regarding the parameter value (P_{SERA}^{+} or P_{DNA}^{+}) and L(X|θ) is the likelihood function for a Binomial process expressing the estimated probability of observing a given number of positive animals given the number of positive animals detected (*s*) in a given population *(n)* with a test characterized by its sensitivity (*Se*) and specificity (*Sp*).
In the formula, f(θ|X) represents the posterior distribution describing the knowledge of P_{SERA}^{+} or P_{DNA}^{+} having observed 's' positive animals in the current study and given our prior knowledge about the value of the parameter before 's' positive animals were observed.

In this hypothetical model we used results from the epidemiological field study of pigs and cattle conducted in the UK as part of this project (Opsteegh, Schares et al. 2016).

Serology (pigs)
n =2071
s =75
Se= (min 73%; max=89%)
Sp= (min 86%; max=98%)

Molecular (cattle)
n =305
s =5
Se= (min 79%; max=99%)
Sp= 100%

Step 2. Proportion of animals with viable cysts.

For the purpose of a quantitative exposure assessment, it is important to discriminate the presence of viable and non-viable organisms. In this step the proportion of animals with viable cysts is

estimated as a function of the diagnostic test results (step 1). . Hence, according to the diagnostic test used (serological or molecular), the probability of a given number of any animals being infected with viable cysts at slaughter (P_{vcysts}) is estimated as a function of the agreement between the detection of antibodies or DNA fragments and demonstration of viable *T. gondii* by bioassay; formally:

$$P_{VCysts} = \left(P_{bioassay^+} \middle| P_{SERA^+}\right) + \left(P_{bioassay^+} \middle| P_{SERA^-}\right)$$

$$or$$

$$P_{VCysts} = \left(P_{bioassay^+} \middle| P_{DNA^+}\right) + \left(P_{bioassay^+} \middle| P_{DNA^-}\right)$$

If serological tests are adopted, $P_{bioassay}^{+}|P_{SERA}^{+}$ and $P_{bioassay}^{+}|P_{SERA}^{-}$ are the conditional probabilities of observing viable cysts (using bioassay tests) in seropositive (P_{SERA}^{+}) or seronegative animals (P_{SERA}^{-}). Similarly, if molecular tests are adopted $P_{bioassay}^{+}|P_{DNA}^{+}$ and $P_{bioassay}^{+}|P_{DNA}^{-}$ are the conditional probability of observing viable cysts in bioassay given the detection of genetic material in PCR.

In this hypothetical model we used results from the pig study conducted in France as part of the EFSA project (*10*)

$P_{VCYTS}|SERA^{+} = 69\% * P_{SERA}^{+}$

$P_{VCYTS}|SERA^{-} = 6\% * P_{SERA}^{-}$

Step 3 Number of viable cysts in T.gondii's predilection tissue.

This step estimates the number of viable cysts (N_{cyst}) in a portion of *T. gondii's* predilection tissue as a function of (i) the number of bradyzoites (N_{br}) detected and (ii) the current knowledge about the number of bradyzoites per cyst (N_{br_cyst}).

According to the evidence of a recent publication (Juránková, Basso et al. 2014) and conclusions of an extensive review conducted by the EFSA consortium (Opsteegh, Schares et al. 2016), the brain appears to be the organ where the parasite is most likely to be detected in pigs. As the distribution describing N_{br} was obtained fitting the results obtained from portions of 100g (see next paragraph), therefore a standard 100g portion of brain is considered in this step for the purpose of the model.

3.1 Number of bradyzoites/100g.

An estimate of bradyzoite concentration in meat was described by Opsteegh et al. in a previous study (Opsteegh, Prickaerts et al. 2011). In that work, the author fitted results of 35 sheep heart samples of 100g analysed with real-time PCR and a *Betageneral* distribution with parameters α_1 of

6.5, α_2 of 5.7, minimum of 0 and maximum of 6.8 was assumed to describe the $\log_{10}$-transformed bradyzoite numbers for 100g samples (N_{br}). In that study, the number of cysts from which the bradyzoites came from was not explored.

3.2 Number of bradyzoites per cyst.

Intuitively, the number of bradyzoites in cysts is related to the size of the cyst itself and several experiments have shown that tissue cysts vary in size as a function of the age (Van der Waaij 1959, Ferguson and Hutchison 1987, Shaapan and Ghazy 2007, Hossein, Parvin et al. 2009). Young tissue cysts may be as small as 5 µm in diameter containing as few as two organisms while older ones may contain hundreds of parasites (Dubey, Lindsay et al. 1998). The size of a mature tissue cyst is variable; up to 70 µm in diameter for cysts of 12 weeks in mice (mean diameter of 100 cysts= 42 µm) were reported by Van der Waaij (1959) while recently, Hossein observed a diameter of more than 100µm for a cyst of the same age (Hossein, Parvin et al. 2009). However, it is important to emphasize that to date, accurate data about the size of mature cysts together with the relative number of bradyzoites they may contain are still lacking with available information being characterized by huge uncertainty. The presence of more than 1,000 bradyzoites was estimated by Dubey et al. in a highly flattened tissue cyst that initially passed through a 63-µm filter (Dubey, Lindsay et al. 1998) while in the mature tissue cyst illustrated by Huskinson-Mark et al. (1991) 990 bradyzoites are clearly visible but the size of the cyst was not reported. Although the validity of the information has been questioned by Dubey et al. (1998), up to 60,000 bradyzoites were observed in a study conducted by Baverley in 1958 (Beverley 1958).

In conclusion, there are no firm data on the number of bradyzoites in a tissue cyst and it is not possible to predict the age (i.e. the size) of the cysts in an infected animal; therefore, for the purpose of the hypothetical model and according to the current knowledge, the number of bradyzoites per cyst was estimated as follows:

Assuming the cysts in the brain are spherical (Dubey, Lindsay et al. 1998) with a wall of 0.5µm thick and diameters ranging from 5 to 100µm as a function of the age and taking the size of a bradyzoite to be 7 x 1.5µm, a number of bradyzoites per cyst ranging from a minimum ($N_{br\_cyst\_MIN}$) of 3 to maximum of 41,000 can be calculated. However, in order to account for the variability and the uncertainty observed in the size of mature cysts, the upper limit of the range ($N_{br\_cyst\_MAX}$) was estimated accounting for the uncertainty surrounding the diameter of mature cysts by the distribution:

$N_{br\_cyst\_MAX}$ = Pert (Min, Max, Most likely) eq.2

Where the parameters are the number of the bradyzoites per cyst calculated using 20µm, 100µm and 60µm as the 'Minimum', 'Maximum' and 'Most likely' diameter for a mature tissue cyst respectively. The minimum and the maximum values were taken from indications reported in literature (Dubey, Lindsay et al. 1998, Hossein, Parvin et al. 2009) while the most likely was obtained as simple mathematical mean.

Number of viable cysts in T. gondii's predilection tissue.

According to the indications described in the previous steps and the demonstrative purpose of the work, the final number of cysts in 100g of predilection tissue was modelled as follows:

$N_{br\_cyst} = Uniform(N_{br\_cyst\_MIN}; N_{br\_cyst\_MAX})$ for $N_{br} > N_{br\_cyst\_MAX}$ eq.3

$N_{br\_cyst} = Uniform(3; N_{br})$ for $N_{br} \leq N_{br\_cyst\_MAX}$ eq.4

This way, the predicted N_{br_cyst} is at least equal to N_{br} (i.e. bradyzoites are into at least 1 cyst) if the predicted number of bradyzoites is equal or lower than $N_{br\_cyst\_MAX}$. For the purpose of the work, it is assumed that all the viable cysts in the infected tissue are at the same growing stage (i.e. all the cysts are of the same size). Therefore, the number of cysts in 100g is estimated as:

$N_{cyst} = N_{br} / N_{br\_cyst}$ eq.5

Step 4. Number of viable cysts/100g in edible tissues.

In this step, the number of cysts in 100g of edible tissue (N_{cyst_Ti}) is estimated as a function of the number of cysts in the predilection tissue. In fact, the number of viable cysts that are present in the predilection tissue is expected to be different from the number of cysts in edible tissue; and even though the ratio coefficient (γ) describing the relationship: $N_{cyst\_Ti}|N_{cyst}$ (i.e. number of cysts in the edible tissue of interest *i* given the number of cysts in the predilection tissue) remains unknown, it is expected to be lower than 1, formally:

$$N_{cyst\_Ti} = \gamma * N_{cyst}$$ eq.6

$$\gamma = N_{cyst\_Ti}|N_{cyst} = Uniform(0; 0{,}99)$$ eq.7

As there is currently no information on the N_{cyst_Ti} : N_{cyst} ratio (γ), to account for the uncertainty regarding the proportion of viable cysts expected in edible tissues given the number of viable cysts recovered in predilection tissues, an hypothetical uniform distribution was used in this model with γ ranging from 0 to 0.99

Outputs and Implications from data gaps

The hypothetical model was run with 100000 iterations to estimate the probability distribution describing the occurrence of viable cysts in a 100g portion of edible tissue (N_{cysts_Ti}) and identify the most influential variables. After simulation, the probability of having at least 1 cyst in 100g of a hypothetical edible tissue (T*i*) was 6.2% using serological test results in pigs in the UK as model input for the true prevalence of infection.

In other words, the predicted number of viable cysts/100g in a given edible tissue ($N_{cysts\_p_{Ti}}$) was greater than zero only above the 95th percentiles of the cumulative distribution (Figure 4).

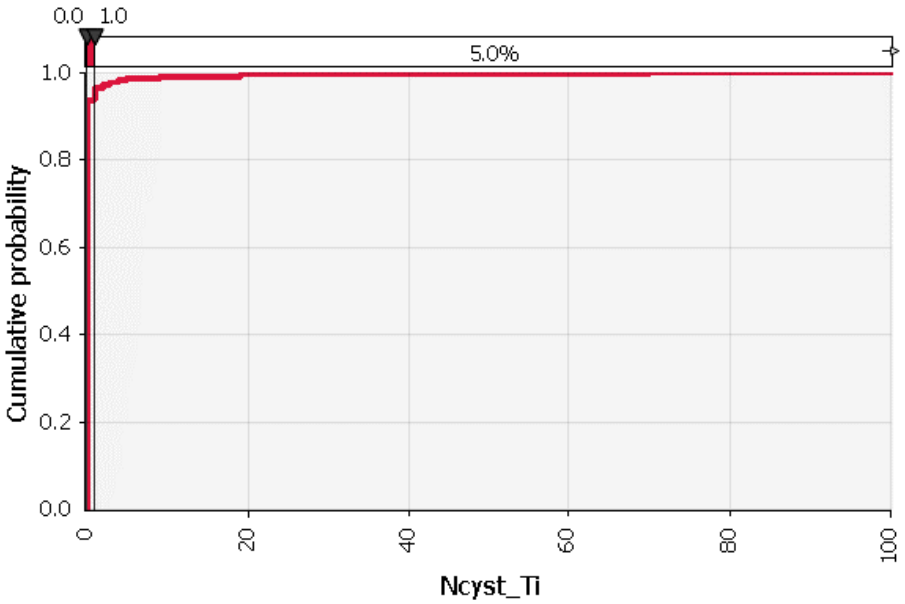

Figure 4 Cumulative distribution of predicted number of cysts in 100g in a given edible tissue (Ti)

As a number of uncertainty distributions were included to account for the lack of evidence surrounding some of the most important parameters, a sensitivity analysis was performed to assess which input variable were more influential in the output; to this end, a tornado chart with input ranked by their effect on the output mean is presented (Figure 5).

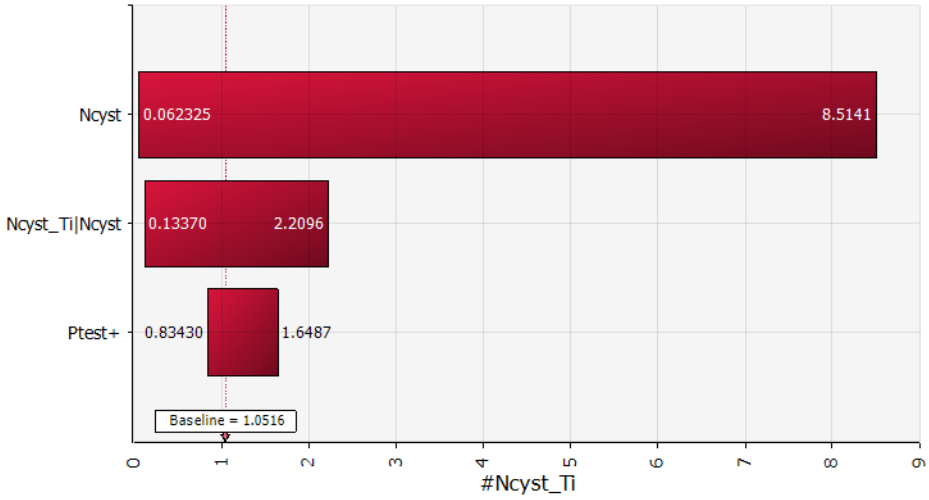

Figure 5 Sensitivity analysis. Inputs ranked by effect on output mean

Moreover, in order to quantify the practical implications of the distributions representing the uncertainty and the lack of data on the model's output, a second order plot is presented (Figure 6). The plot represents the results of 10 simulated scenarios in which all the uncertainty distributions that are directly (i.e. P_{test}, and γ) or indirectly (i.e. $N_{br\_cyst\_MAX}$) used in the model were kept fixed to one randomly selected percentile (1 scenario = 1 percentile). However, amongst those randomly generated scenarios, two extreme combinations were arbitrary assessed:

- Scenario1: a scenario in which all the uncertainty distributions are kept fixed to the value corresponding to their 5^{th} percentile.
- Scenario2; a scenario in which all the uncertainty distributions are kept fixed to the value corresponding to their 95^{th} percentile.

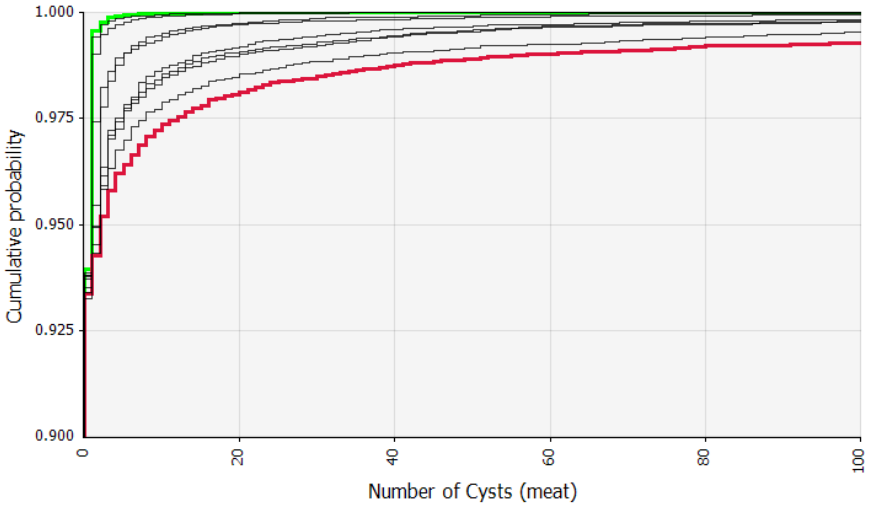

Figure 6 Second order plot representing the effect of the uncertainty surrounding the model inputs in the computation of the cumulative distribution describing the number of cyst in 100g of pork meat. Red and green distributions represent the outputs obtained for Scenario1 and Scenario2 respectively.

The final number of viable cysts per 100g in infected edible products ranged from 1 to 40 at the 99th percentile of simulated scenarios and from 1 to 61 when the best and the worst scenarios respectively were simulated.

In summary, the hypothetical model we conceptualized highlights how some of the most important parameters and relationships of the variables involved are still characterized by limited or totally missing information. In particular, figures 5 and 6 clearly illustrate that with the current level of understanding, results from a formal quantitative risk assessment would not provide any valid information for policymakers with respect to the exposure of humans to *T. gondii* through consumption of meat products.

Quantitative risk assessment models should be as simple as possible but not simplistic, otherwise, the biological dynamics of the systems are not captured and results are not reliable. Although the lack of data characterizing even the basilar key inputs still prevents a valid assessment of the exposure, the model we have developed represents an important integration of that described by Opsteegh et al. (2011). In fact, having structured the model for the assessment of the exposure to *T. gondii's* cysts rather than the bradyzoites, the model is not only an improved representation of the biological system but also allows the assessment of exposure to the parasite through consumption of products that are made with tissue from different (infected and not infected) animals.

It should be noted that beyond the assessment of the exposure, the lack of dose-response information for humans represents an important limitation to the final assessment of the risk for human health through consumption of meat containing one or more viable cysts. However, as the

model allows for an estimation of the number of bradyzoites ingested as a function of the number of cysts (dotted line in figure 6); it can be easily extended beyond the assessment of the exposure when Information to fill this key data gap becomes available.

CONCLUSIONS

- Assessment of the potential role of the foodborne route for *T. gondii* infection in humans requires knowledge of the frequency of infection in meat-producing animals and the concordance between infection and presence of viable cysts in meat. Results from previous studies suggest that the level of concordance between serology and presence of viable cysts in meat differs across livestock species. Studies carried out as part of the EFSA consortium project found a fairly good agreement in pigs (69.5% detection of viable cysts in seropositive animals) and poultry (85.7% detection of viable cysts in seropositive animals) which is aligned with results previously reported in the literature. On the other hand, agreement is poor in cattle and horses limiting the usefulness of serological data to infer prevalence of infection and risk for the consumer. While serological tests are useful to estimate prevalence of *T. gondii* infection in pigs, sheep and poultry, estimation of the prevalence of *T. gondii* infection in the cattle population should be based on diagnostic tests detecting *T. gondii* DNA or viable cysts. In cattle, serological results should not be used as a proxy for presence of cysts in meat that would enter the food chain.
- The presence of cats and cat's faecal contamination have been repeatedly found to increase the risk of infection in all livestock species. This was also true in UK pig farms studied where the risk of *T. gondii* infection increase 2.6 fold in those farms where cats could get in direct contact with pigs' feed. Prevention of *T. gondii* infection in pigs should therefore be based on avoiding food contamination with cat's faeces.
- Although extrapolations and comparisons should be made with caution given the non-probabilistic selection of farms and different survey methodologies applied in different countries, the results from studies carried out in the UK suggest a relatively low level of infection in cattle and pigs raised and slaughtered in the country, with no clear geographic pattern of positive animals. Once adjusted for the number of animals tested per batch and the Sensitivity and Specificity of the test, the adjusted herd level prevalence for pigs farms sampled was 11.5% with most positive animals coming from a few farms. In cattle, the number of positive animals tested in abattoir was low (less than 2% of tested animals) and the test results suggest some variation on the parasite load of positive animals.
- Estimates of the relative importance of different meat products as a source of human exposure to *T. gondii* have been made available for Australia, The Netherlands and the US. However, they are based on a number of critical assumptions for which data available are very limited. Specifically, the number and distribution of viable cysts in meat and tissues of

infected animals and the number of bradyzoites per cyst remains an important knowledge gap. Simulated scenarios, using uninformative distributions in parameters in which data are not available, generated very broad range of estimates on the number of cysts present in edible tissue. Dose-response parameters in humans and the potential effect of different meat processes on reducing bradyzoite load and/or inactivating viable cysts also remains unknown. Generating knowledge to fulfil these data gaps is essential to develop a sound quantitative assessment and quantifying the relative contribution of meat to human *T. gondii* infection. In the meantime, estimates of numbers of cases of toxoplasmosis attributed to meat consumption should be interpreted with caution.

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REFERENCES

AMCSF (2012). Ad Hoc Group on Vulnerable groups: Risk profile in relation to toxoplasma in the food chain. Advisory committe on the microbiological safety of food.

Andreoletti, O., H. Budka, S. Buncic, P. Colin, J. D. Collins, A. De Koeijer, J. Griffin, A. Havelaar, J. Hope, G. Klein, H. Kruse, S. Magnino, A. Martinez Lopez, J. McLauchlin, C. Nguyen-The, K. Noeckler, B. Noerrung, M. Prieto Maradona, T. Roberts, I. Vagsholm and E. Vanopdenbosch (2007). "Surveillance and monitoring of *Toxoplasma* in humans, food and animals. Scientific opinion of the Panel on Biological Hazards." The EFSA journal **583**: 1-64.

Batz, M. B., S. Hoffmann and J. G. Morris (2011). Ranking the risks: the 10 pathogen-food combination with the greatest burden on Public Health. Emerging Pathogens Institute. USA, University of Florida.

Beverley, J. (1958). "A rational approach to the treatment of toxoplasmic uveitis." Transactions Ophthal. Soc. **78**: 109-121.

Cook, A. J. C., R. E. Gilbert, W. Buffolano, J. Zufferey, E. Petersen, P. A. Jenum, W. Foulon, A. E. Semprini and D. T. Dunn (2000). "Sources of *Toxoplasma* infection in pregnant women: European multicentre case control study." BMJ.

Dubey, J. P. and G. Desmonts (1987). "Serological responses of equids fed Toxoplasma gondii oocysts." Equine Veterinary Journal **1987**: 337-339.

Dubey, J. P., H. R. Gamble, D. Hill, C. Sreekumar, S. Romand and P. Thulliez (2002). "High prevalence of viable *Toxoplasma gondii* infection in market weight pigs from a farm in Massachusetts." Journal of Parasitology **88**: 1234-1238.

Dubey, J. P., D. S. Lindsay and C. A. Speer (1998). "Structures of *Toxoplasma gondii* Tachyzoites, Bradyzoites, and Sporozoites and Biology and Development of Tissue Cysts." Clinical Microbiology reviews: 267-299.

EFSA (2015). "The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013." EFSA Journal, **13**(1).

Ferguson, D. J. and W. M. Hutchison (1987). "An ultrastructural study of the early development and tissue cyst formation of *Toxoplasma gondii* in the brains of mice." Parasitology Research **73**: 483-491.

Flatt, A. and N. Shetty (2012). "Seroprevalence and risk factors for toxoplasmosis among antenatal women in London: a re-examination of risk in an ethnically diverse population." European Journal of Public Health **23**: 648-652.

Gamble, H. R., J. P. Dubey and D. N. Lambillotte (2005). "Comparison of a commercial ELISA with the modified agglutination test for detection of *Toxoplasma* infection in the domestic pig." Veterinary parasitology **128**: 177-181.

Guo, M., R. L. Buchaman, J. P. Dubey, D. E. Hill, D. N. Lambillotte, Y. Ying, H. R. Gamble, J. L. Jones and A. K. Pradhan (2015). "Qualitative Assessment for *Toxoplasma gondii* Exposure Risk Associated with Meat Products in the United States." Journal of Food Protection **78**: 2207-2219.

Havelaar, A. H., J. A. Haagsma, M. Mangen, J. M. Kemmeren, L. P. B. Verhoef, S. M. C. Vijgen, M. Wilson, I. H. M. Friesema, L. M. Kortbeek, Y. T. H. P. van Duynhoven and W. van Pelt (2012). "Disease burden of foodborne pathogens in the Netherlands." International Journal for Food Microbiology **156**: 231-238.

Hill, D. E., S. Chirukandoth, J. P. Dubey, J. K. Lunney and H. R. Gamble (2006). "Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine." Veterinary parasitology **141**: 9-17.

Hossein, H., R. Parvin and A. Mohsen (2009). "Study on growth of *Toxoplasma gondii* tissue cyst in laboratory mouse." Jundishapur Journal of Microbiology: 140-143.

Huskinson-Mark, J., F. G. Araujo and J. S. Remington (1991). "Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*." Journal of Infectous Diseases **164**: 170-177.

Juránková, J., W. Basso, H. Neumayerová, V. Baláž, E. Jánová, X. Sidler, P. Deplazes and B. Koudela (2014). "Brain is the predilection site of Toxoplasma gondii in experimentally inoculated pigs as revealed by magnetic capture and real-time PCR." Food Mocrobiology **38**: 167-170.

Mie, T., A. M. Pointon, D. R. Hamilton and A. Kiermeier (2008). "A qualitative assessment of *Toxoplasma gondii* risk in ready-to-eat smallgoods processing " Journal of Food Protection **71**: 1442-1452.

Montoya, J. G. and O. Liesenfeld (2004). "Toxoplasmosis." Lancet **363**: 1965-1976.

Opsteegh, M., M. Langelaar, H. Sprong, L. den Hartog, S. De Craeye, G. Bokken, D. Ajzenberg, A. Kijlastra and J. van der Giessen (2010). "Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR." International Journal for Food Microbiology **139**: 193-201.

Opsteegh, M., M. Maas, G. Schares, J. Van der Giessen and on behalf of the consortium (2016). Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01). An extensive literature review. Final report. EFSA supporting publication, EFSA.

Opsteegh, M., S. Prickaerts, K. Frankena and E. G. Evers (2011). "A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands." International Journal for Food Microbiology **150**: 103-114.

Opsteegh, M., G. Schares, R. Blaga, J. Van der Giessen and on behalf of the consortium (2016). Experimental studies on *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01). Final report. EFSA supporting publication, EFSA.

Opsteegh, M., P. Teunis, L. Zuchner, A. Koets, M. Langelaar and J. Van der Giessen (2011). "Low predictive value of seroprevalence of *Toxoplasma gondii* in cattle for detection of parasite DNA." International Journal for Parasitology **41**: 343-354.

Shaapan, R. and A. Ghazy (2007). "Isolation of *Toxoplasma gondii* from horse meat in Egypt." Pakistan journal of biological sciences **10**: 174-177.

Van der Waaij, D. (1959). "Formation, growth and multiplication of *Toxoplasma gondii* cysts in mouse brains." Tropical and Geographical Medicine **11**: 345-360.

ANNEXES

Annex 1. Recording sheets used in the multi-country cattle study

Cattle study – Correlation between serology bioassay and PCR-based detection of *Toxoplasma gondii*

Recording sheet

Slaughterhouse_____ **Date of sampling**: _____

Sample collection by: _____ **Data recording by**: _____

Blood collected at: □bleeding □evisceration

Animal sampled	**Animal ID**	**Last two digits of the ear tag**	**Age of the animal**	**Sex** *(tick as appropriate)*	**Breed or type** *(tick as appropriate)*
1	B001-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
2	B002-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
3	B003-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
4	B004-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
5	B005-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
6	B006-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed
7	B007-			☐ Male ☐ Female	☐ Dairy ☐ Beef ☐ Cross-breed

NOTE: Please verify in the intake sheets that the animals sampled are from different farms.

Once sampling is completed please place the recording sheet in the plastic envelop provided and put it in the box to be posted to the Royal Veterinary College

Annex 2. Recording sheets used in the UK cattle study

Level of *T. gondii* infection in cattle in the UK

Recording sheet

Slaughterhouse:_____ Date of sampling:_____

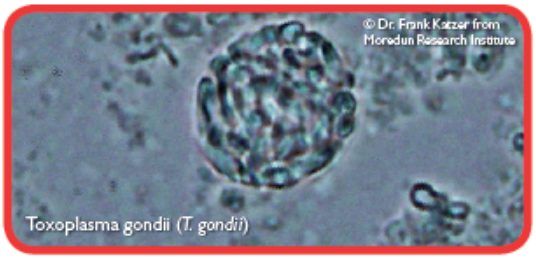
Animal ID	Ear tag	Notes
C001		
C002		
C003		
C004		
C005		
C006		
C007		
C008		
C009		
C010		
C011		
C012		
C013		
C014		
C015		
C016		
C017		
C018		
C019		
C021		
C022		
C023		
C024		
C025		
C026		
C027		
C028		
C029		
C030		

NOTE: Please verify in the intake sheets that the animals sampled are from different farms.

Once sampling is completed please place the recording sheet in the plastic envelop provided and put it in the box with the diaphragm samples

Annex 3. Note published in the British Pig Executive (BPEX) newsletter

NEW field studies on *Toxoplasma gondii* in the UK



The European Food Safety Authority (EFSA) has identified Toxoplasma as one of the priority targets for official controls in pig meat.

The EFSA and The Advisory Committee on the Microbiological Safety of Food (ACMSF) of the Food Standards Agency (FSA) have both highlighted that more data is needed on Toxoplasma and, in particular, the incidence, severity and main sources of infection in the human population. It also wants to confirm its prevalence in carcases and the extent to which meat consumption contributes to human infection.

The frequency and infectivity of *T. gondii* cysts in meat and how this relates to seroprevalence in animals has been identified as an important data gap across European countries (including the UK).

To improve knowledge of Toxoplasma, a consortium of seven EU countries has been organised by the EFSA to investigate the presence of *T. gondii* in the meat of the main meat producing animals (cattle, pigs, sheep, horses and poultry) and to identify possible farm-level risk factors.

The research in the UK has been funded by FSA and carried out by the Royal Veterinary College and Moredun Research Institute. The researchers are conducting laboratory investigations and field studies focusing on cattle and pigs.

The field study in pigs involves a survey using a questionnaire and testing blood samples taken at slaughter for *T. gondii* on a subset of pigs. Pig farmers and slaughterhouses are going to be contacted shortly asking for their participation.

The results from this study will fill some of the key knowledge gaps that currently preclude a formal assessment of potential control options to mitigate the risk of foodborne exposure to *T. gondii* to make UK pork even safer for UK consumers.

We welcome your participation as the results will ensure that up to date evidence is available for the UK to negotiate possible EU interventions in the future.

If you are interested in taking part or you want further information please contact Georgina Limon at **glimon@rvc.ac.uk**

BPEX contact

For any queries please contact:
Ouafa Doxon, Health Information and Food Safety Coordinator,
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Email: Ouafa.Doxon@bpex.ahdb.org.uk
Website: www.bpex.org.uk

Background to *Toxoplasma gondii*

Toxoplasmosis is a zoonosis caused by a parasite called *Toxoplasma gondii* (*T. gondii*). The life cycle of *T. gondii* includes cats as the definitive host and mammals and birds as intermediate hosts. Humans can get infected via different routes but the major source of *T. gondii* infections in Europe and North America is from eating raw or undercooked meat or meat products containing infective cysts.

According to the European Centre for Disease Prevention and Control (ECDC), human infections do not usually result in any symptoms or in some cases they can show as mild flu-like symptoms. The ACMSF estimates that 350,000 people become infected with toxoplasma each year in the UK, of which 10-20% are symptomatic. Scientists in the Netherlands suggest that Toxoplasma could cause the highest disease burden amongst well known foodborne pathogens. However, the true burden of toxoplasma could be largely underestimated because of under-reporting.

Farm animals get infected via ingestion of soil, water and grass contaminated with faeces from infected cats.

Annex 4. Questionnaire used in the cross sectional pig study

On-farm risk factors for *Toxoplasma gondii* infection in pigs

This study aims to obtain insights into possible farm-level characteristics that increase/decrease the likelihood of *T. gondii* infection, allowing a better understanding of the UK situation.

We will send you a report with the result of the sampling and the main findings, at the end of the study, to be discussed with your veterinary surgeon.

Please note that information you provide will be kept **strictly confidential** and results of this study will not be linked to your name. If you have any question please contact Georgina Limon at glimon@rvc.ac.uk

Please return the filled-in questionnaire using the pre-paid envelope provided.

Farm ID: _____ **SLAPMARK**_____

Owner's name	
Farm address	
Post code	
Telephone	
e-mail	
Marketing group	
Date	

CONSENT AGREEMENT

I agree to participate in this study. I have been given the opportunity to ask questions and received satisfactory answer.

I further agree for blood samples taken at the slaughterhouse to be tested for respiratory pathogens and *Toxoplasma gondii.*

Signature _____ Date:_____

SECTION 1 - FARM CHARACTERISTICS

Please answer the following questions by ticking the most appropriate answer, unless otherwise specified.

1. **Which of the following best describes the production cycle in the farm?**

☐ Farrow to finish

☐ Breeding to weaning

☐ Weaning to finishing

☐ Grower to finishing

☐Other. *Please specify*: _____

1.1. **If *weaning to finishing* OR *grower to finishing*, where did you get the pigs from the last batch sent to the slaughterhouse?**

☐From a unit placed in another site but part of the same farm (same owner)

☐From another farm (different owner)

☐From different farms

☐Other, *please specify*: _____

1.2. Do you always get the pigs from the same place?

☐Yes, always from the same place

☐No, it varies where we get the pigs from

2. **Do you keep pigs in more than one site/holding?**

☐Yes ☐No

2.1. *If yes*, **how many sites?** _____

3. **What is the production system in the farm?**

☐ All in all out

☐ By farm

☐ By site

☐ By building

☐ By pen

☐ Continuous

☐ Other: *Please specify:* _____

4. **Using the definitions provided below, please complete the table by ticking the box that best describes the way animals are kept in the farm (outdoor/indoor/both).** If

certain production stage is not kept on the farm please write N/A in the observations.

Indoors is defined as keeping pigs in enclosed buildings (i.e. delimited by solid walls) and pigs are not able to go outside the building.

Outdoors is defined as kept in the field within defined boundaries where they are free to roam and are provided with food, water and shelter.

PRODUCTION STAGE	Kept outdoor (all the time)	Kept indoor (all the time)	Kept part of the time outdoor and part of the time indoor	Observations
Dry sows	☐	☐	☐	
Lactating sows	☐	☐	☐	
Boar	☐	☐	☐	
Piglets	☐	☐	☐	
Weaners	☐	☐	☐	
Growers	☐	☐	☐	
Finishers	☐	☐	☐	

PLEASE ANSWER THE REMAINDING QUESTIONS, CONSIDERING ONLY THE SITE FROM WHERE THE LAST BATCH OF PIGS SENT TO SLAUGHTER CAME FROM.

5. **Please fill in the table below indicating the total number of pigs for each production stage at this moment.**

Number of pigs							
Sows (Dry and Lactating)		Boar	Piglets	Weaners	Growers	Finishers	Total number

Piglets <3weeks or <8Kg; ***Weaners*** 3-10 weeks or 8-30Kg; ***Growers*** 11-14 weeks or 30-50kg; ***Finishers*** >15weeks or >50 Kg

6. **Are there other livestock species (apart from pigs) in this site?**

☐Yes ☐No

***6.1. If yes,* which species?** *Tick all that apply*

☐Cattle Number of animals_____

☐Sheep Number of animals_____

☐Goats Number of animals_____

☐Poultry Number of animals_____

☐Other: Please specify _____

SECTION 2 - FOOD AND WATER

Please answer the following questions by ticking the most appropriate answer unless otherwise specified.

7. **What is the type of food used in this site?** *Tick all combinations that apply*

	Home mix	Purchase compound	By product	Observations
Dry	☐	☐	☐	
Wet	☐	☐	☐	
Both	☐	☐	☐	

8. **Where is the animal feed stored?** *Tick all that apply*

☐ Open silo ☐ Close silo

☐ Open storage ☐ Close storage

☐ Bags for food

☐ Other: *Please specify* _____

9. **Which types of feeders are used in this site?** *Tick all that apply*

☐ None (floor) ☐ Individual feeders

☐ Bowl ☐ Pipeline

☐ Dump feeders

☐ Other. *Please specify:* _____

10. Where does the pigs' drinking water come from? *Tick all that apply*

☐ Main supply (community tap water)

☐ Local canal / stream

☐ Well

☐ Other. *Please specify:* _____

11. How is water supplied to pigs? *Tick all that apply*

☐ Nipple ☐Trough

☐ Cups ☐ Other: *Please specify:* _____

SECTION 3 – BIOSECURITY

12. **What is normally the down time (in days) between batches?** (*If the farm has a continuous system please put NA and go to question 15*)
_____ days

13. Is it common practice to <u>clean</u> between batches?

☐Yes, it is <u>always</u> cleaned between batches

☐Yes, <u>most of the times</u> is cleaned between batches

☐Rarely

If yes, **what system is used for cleaning?**

☐ Muck out

☐ High pressure washing

☐ Wet cleaning

☐ Other: *Please specify:*

_____

14. Is it common practice to <u>disinfect</u> between batches?

☐Yes, it is <u>always</u> disinfected between batches

☐Yes, <u>most of the times</u> is disinfected between batches

☐Rarely

If yes, **which disinfectant is used?**

_____

15. Are staff designated to work <u>exclusively</u> in certain areas of this site?

☐Yes ☐No

16. **Please indicate which of the following is provided in this site** (*Tick all that apply*)

☐Protective clothing for workers

☐Protective clothing for visitors

☐Protective footwear for workers

☐Protective footwear for visitors

☐Boot dips

☐Dip for vehicles

☐Hand washing before entering the farm

☐Other: Please specify

17. **Is there a <u>public path</u> running through or sharing boundaries with this site?**

PRODUCTION STAGE		**Observations**
Dry sows	☐Yes ☐No ☐N/A	
Lactating sows	☐Yes ☐No ☐N/A	
Boars	☐Yes ☐No ☐N/A	
Weaners	☐Yes ☐No ☐N/A	
Growers	☐Yes ☐No ☐N/A	
Finishers	☐Yes ☐No ☐N/A	

N/A = Not applicable (i.e. this site does not have this production stage)

18. Do you keep cats in this site?

☐Yes ☐No

If NO, go to question 19

18.1. *If yes*, **how many cats? _____**

18.2. **What is the main reason for keeping cats?**

☐ Rodent control

☐ Pets

☐ Other. *Please specify*: _____

19. Is it possible that cats <u>not belonging to this site</u> get into the site?

☐Yes ☐No ☐Not sure

20. Is it possible that cats come into direct contact with the pigs?

☐ <u>Yes</u>, cats <u>definitely</u> come into direct contact with pigs

☐ <u>Yes</u>, it is <u>very likely</u> that cats come into contact with pigs

☐ <u>No</u>, cats cannot come into contact with pigs

☐ Not sure

21. Is it possible that cats come into contact with pigs' food?

☐ <u>Yes</u>, cats <u>definitely</u> come into direct contact with pigs' food

☐ <u>Yes</u>, it is <u>very likely</u> that pigs come into contact with pigs' food

☐ <u>No</u>, cats cannot come into contact with pigs' food

☐ Not sure

22. Is it possible that cats come into contact with pigs' drinking water?

☐ <u>Yes</u>, cats <u>definitely</u> come into direct contact with water that will be drunk by pigs

☐ <u>Yes</u>, it is <u>very likely</u> that pigs come into contact with water that will be drunk by pigs

☐ <u>No</u>, cats cannot come into contact with water that will be drunk by pigs

☐ Not sure

SECTION 4 - PREVENTIVE MEDICINE

If the farm keeps animals in more than one site please fill in following table considering all sites.

23. **Please complete the table below concerning the routine vaccination and de-worming used on the farm**

PRODUCTION STAGE	Vaccination	Wormers	
	Vaccine *(tick all that apply)*	Product used	Frequency
Dry sows	□PCV2 □PRRS □Mycoplasma □Parvovirus □Other:		□Once a year □Twice a year □Other:
Lactating sows	□PCV2 □PRRS □Mycoplasma □Parvovirus □Other:		□Once a year □Twice a year □Other:
Weaners	□PCV2 □PRRS □Mycoplasma □Parvovirus □Other:		□Once a year □Twice a year □Other:
Growers	□PCV2 □PRRS □Mycoplasma □Parvovirus □Other:		□Once a year □Twice a year □Other:
Finishers	□PCV2 □PRRS □Mycoplasma □Parvovirus □Other:		□Once a year □Twice a year □Other:

Thank you for your time!