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Cross sectional study of *Toxoplasma gondii* infection in pig farms in England

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Abstract

Ingestion of undercooked meat has been proposed as an important source of human *T. gondii* infection. To ascertain the contribution of meat consumption to the risk of human infection, estimates of the prevalence of infection in meat-producing animals are required. A cross sectional study was conducted to assess *T. gondii* infection in pigs raised in England, to identify risk factors for infection and to compare performance of two serological tests: modified agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA).

Blood samples from 2071 slaughter pigs originating from 131 farms were collected and 75 (3.6%) were found positive by MAT. Positive pigs originated from 24 farms. A subset of samples (n=492) were tested using ELISA, and a significant disagreement ($p<0.001$) was found between the two tests. An empirical Bayes approach was used to estimate the farm-level prevalence and the probability of each individual farm having at least one positive animal considering the uncertainty arising from the sampling strategy and the imperfect test performance. The adjusted farm-level prevalence was 11.5% (95% credible interval of positive farms 8.4%-16.0%). Two different criteria were used for classifying farms as infected: (i)≥50% probability of having at least one infected pig (n=5, 6.8%); (ii)≥10% probability (n=15, 20.5%). Data on putative risk factors was obtained for 73 farms. Using a 10% cut-off, the relative risk (RR) of infection was higher on farms where cats have direct access to pigs' feed (RR=2.6; p=0.04), pigs have outdoor access (RR=3.0; p=0.04) and farms keeping ≤200 pigs (RR=3.9; p=0.02), with strong collinearity between the three variables.

The findings suggest a low level of *T. gondii* infection in the farms studied, most of which are likely to send to slaughter batches composed of 100% uninfected pigs. These results provide key inputs to quantitatively assess the *T. gondii* risk posed by pork to consumers.

Introduction

Toxoplasmosis is a worldwide distributed zoonosis caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*). Most warm-blooded animals can be infected and act as intermediate hosts in the life-cycle of the parasite. Felines are the definitive host and the only species able to excrete sporulated oocysts in faeces potentially contaminating the environment, soil and crops (Montoya and Liesenfeld, 2004).

Humans can become infected via three main routes: (i) congenital, (ii) ingestion of sporulated oocysts present in cats' litter trays or contaminated soil, water and vegetables and (iii) consumption of raw or undercooked meat containing *T. gondii* bradyzoites clustered in tissue cysts ('infective cysts') (Andreoletti et al., 2007; Tenter et al., 2000). The latter has been considered the most important route of infection in developed countries by the World Health Organization (WHO, 2015). It is estimated that up to a third of the world's population is currently infected with *T. gondii* with important differences between and within countries (Pappas et al., 2009; Tenter et al., 2000). In recent years, *Toxoplasmosis* has been ranked as posing the highest disease burden among foodborne pathogens in Europe (Havelaar et al., 2012; WHO, 2015), and consumption of pork has been ranked second among the top 10 pathogen-food combinations in the US (Batz et al., 2011). Estimates of the overall incidence of human toxoplasmosis in England are lacking, as records of the number of confirmed cases (on average 330 cases per year) represent a small proportion of the total number of cases in the population given the asymptomatic nature of the infection in healthy individuals (PHE, 2015, 2016). On the contrary, immunocompromised people can become seriously ill, whilst infection during pregnancy could result in lifelong complications for the offspring (Andreoletti et al., 2007).

Pigs rarely show clinical signs when infected with *T. gondii* and detection of *T. gondii* cysts during meat inspection is not feasible given their microscopic size. Numerous techniques are available for antibody detection and a fairly good correlation has been reported in pigs between seropositivity and presence of cysts (Dubey et al., 2002; Gamble et al., 2005; Hill et al., 2006). Therefore presence of antibodies can be used as an indicator for the potential presence of infective cysts in pork. Among the serological tests available, the modified agglutination test (MAT) has the highest sensitivity and specificity (based on isolation of viable *T. gondii* from tissues of experimentally-infected pigs as gold standard) having the advantage of not being affected by cross-reactivity with other parasites (Dubey, 1997; Dubey et al., 1996; Dubey et al., 1997). In field conditions however, the limited number of studies have reported inconsistent results. A study conducted in naturally infected sows found higher sensitivity and specificity in MAT compared with enzyme-linked immunosorbent assays (ELISA) (Dubey et al., 1995); whilst the contrary was found in a study conducted in finishing pigs (Gamble et al., 2005).

The prevalence of toxoplasmosis in pigs varies between countries and is mainly associated with the presence of cats and contamination of pigs' feed with cat faeces with differences in risk found depending on the type of housing and production system (Assadi-Rad et al., 1995; Garcia-Bocanegra et al., 2010a; Garcia-Bocanegra et al., 2010b; Guo et al., 2016; Kijlstra et al., 2004; Klun et al., 2006; Ortega-Pacheco et al., 2013; Tao et al., 2011; van der Giessen et al., 2007; Weigel et al., 1995). It has been hypothesized that recent trends in consumer habits in developed countries, with a shift towards the consumption of free range and organic pork, where animals have a higher risk of exposure to *T. gondii* from the environment, may result in a higher risk of consumer exposure to *T. gondii* (Kijlastra et al., 2009; van der Giessen et al., 2007).

Policies to mitigate the risk of foodborne exposure to *T. gondii* should be based on scientific risk assessment and best available data. Lack of information regarding prevalence and risk factors for *T. gondii* infection of pigs reared in the UK have been highlighted as important data gaps for the assessment of the risk of pork to human infection (AMCSF, 2012). A recent UK survey in slaughtered pigs (Powell et al., 2016) found that 7.7% of pigs were sero-positive by Sabin-Feldman Dye test (a test that detect *T. gondii* IgG antibodies); potential risk factors for *T. gondii* infection were not assessed. Ideally, prevalence estimation should take into account the imperfect performance of the test and the sampling strategy used.

The objectives of this study were (i) to assess, by means of an empirical Bayes estimation, the probability of *T. gondii* infection in selected commercial farms in England, (ii) to identify factors associated with a higher risk of *T. gondii* infection at farm level and (iii) to compare the performance of the reference serological test for *T. gondii* in pigs (MAT), with a commercially available ELISA.

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. A batch was defined as a group of pigs received in the abattoir from the same herd and on a given day. A note explaining the aim of the study was published in the British Pig Executive (BPEX) newsletter in December 2014 and five commercial slaughterhouses volunteered to take part in the study; they varied in size and throughput from 40 to >10,000 pigs processed per week. Farmers regularly sending pigs to these slaughterhouses were contacted and invited to participate.

The target sample size was calculated as 129 batches in order to be able to estimate prevalence at the level of the batch (expected to be 25%) with 7.5% precision and 95% confidence. In the absence of

farm-level prevalence estimates in England, values reported in other European countries were used as reference (Steinparzer et al., 2015; van der Giessen et al., 2007). Within each batch, the number of pigs needed to be sampled to classify, with 90% confidence, the study batches in 3 groups based on within-batch prevalence (<7.5%; 7.5-25%; >25%) was estimated as 25 pigs.

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

Samples and data collection

Each slaughterhouse was visited up to five times. On the day of the visit, batches of pigs from farmers who agreed to participate were included (in later visits farms already sampled were excluded). From each batch, blood samples were collected from individual pigs during routine slaughter at the point of bleeding (sticking). Nine ml of blood was collected from each pig using pre-labelled vacutainer tubes. 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 sex were recorded.

Information on farm characteristics, management practices and biosecurity were gathered using a standardised questionnaire designed based on a putative risk factors identified in a literature review (Opsteegh et al., 2016). The questionnaire was either sent by post (with a pre-paid envelope to be posted back) or handed directly to farmers at the slaughterhouse. Copies of the questionnaire are available from the corresponding author upon request.

Serology

Blood samples were centrifuged to separate sera from blood cells and sera samples were stored at -20°C until testing using MAT for the detection of *T. gondii* specific immunoglobulin (IgG). Testing was performed at the French Agency for Food, Environmental and Occupational Health and Safety in Reims, France, as previously described (Dubey and Desmonts, 1987). A sample was considered positive if the titre was ≥1:25 (Dubey, 1997). Titres between 1:1 and 1:10 were classified as suspicious.

All MAT-positive and suspicious samples from which sera were available (n=152), plus a subset of 340 samples randomly selected among all the negative (n=1916) with maximum three negative samples per farm, were tested in duplicate by a commercially available ELISA (ID Screen® toxoplasmosis indirect multi-species) according to manufacturer's instructions. The optical density (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% was considered positive, ≤ 40% 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 the repeated test results were also contradictory the sample was considered inconclusive.

McNemar's Chi-squared test for paired data was used to assess whether there was a significant difference in the proportion positive between MAT and ELISA excluding inconclusive results. Repeatability between ELISA results was measured using the coefficient of variation (CV). Low values indicate high precision while the opposite is true for high values. A CV up to 0.20 can be expected due to random variation (Reed et al 2002) and considered acceptable. The CV of each sample was calculated for all the replicate values and then averaged across all 492 samples.

Data analysis

Descriptive statistics were obtained at animal level for all pigs sampled (n=2071) and at farm level for farms which completed the questionnaire (n=73).

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

Intra-farm correlation (ICC) for positive status of individual pigs was estimated using the farm variance (σ) from the mixed effect model considering the farm as a random effect (Wu et al., 2012).

$$ICC = \frac{\sigma^2}{\sigma^2 + \pi^2/3}$$

An empirical Bayes model was used to estimate the farm-level prevalence (Beauvais et al., 2016). Briefly, the probability of each farm having at least one true positive pig was estimated after taking into account the number of pigs tested, how many of them were found to be positive, the imperfect sensitivity and specificity of the test, the uncertainty arising from sampling only a proportion of animals on each farm and "prior" information about the within-farm prevalence probability distribution. The within-farm prevalence probability distribution was generated empirically from this study and does not therefore rely on prior knowledge about the distribution of the disease. 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. MAT sensitivity and specificity of 86% and 95% respectively, were used as inputs (Gamble et al., 2005).

Model results were used to classify farms as positive or negative using two cut-offs: positive farms 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).

In addition, to explore whether there was a difference on the number of farms deemed positive depending on the serological test used, the probability of a farm having at least one true positive pig was estimated using results from the subset of samples tested in duplicate by MAT and ELISA. ELISA sensitivity and specificity of 89% and 98% respectively were used (Gamble et al., 2005).

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. The re-categorisation of variables is described in Table 1.

Crude associations between predictor variables (table 1) and farm status were tested by Fisher's exact or Pearson's Chi squared test as appropriate; relative risk (RR) 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 in the model for further multivariable analysis. Logistic regression was used to assess the relationship between the individual predictor variables and the outcome, accounting for the potential confounding effect of other variables. Odds ratios (OR) obtained from the logistic regression were converted to Relative Risk: $RR=OR/ (1-p_0 + (p_0 * OR))$, where p_0 was the baseline risk (i.e. the risk of being positive in the control group) (Grant, 2014). Note that risk factors were collected retrospectively and therefore, exposure to a given risk factor might have happened after infection. In that cases the relative risk would have been overestimated.

Statistical analyses was performed in R 3.0 (R Development Core Team, 2015) using packages epicalc (Chongsuvivatwong, 2010) and lme4 (Bates et al., 2013).

Results

A total of 2071 pigs from 131 farms were sampled; including 1101 females (53.6%) and 953 (46.3%) males (sex was not recorded for 17 pigs). Antibodies against *T. gondii* by MAT were found in 155 pigs (7.5%) but only 75 pigs (3.6%) had titres ≥1:25 (Figure 1). Sex was not significantly associated with *T. gondii* sero-status (p=0.14).

A higher number of samples were classified as positive using MAT (73 samples were positive by MAT and 37 by ELISA) and the difference was statistically significant (p=<0.001) (Table 2; Figure

S1.1 supplementary material), suggesting serious disagreement between the two tests. For repeated samples, the mean CV values for ELISA were 0.62, therefore there was substantial variation and low precision of the test.

The proportion of farms deemed positive (i.e. farm-level prevalence) was 1.5% higher using results given by ELISA when considering a ≥50% cut-off. However, the opposite happened when considering a 10% cut-off, with more farms deemed positive using results given by MAT (Table S2.1 and S2.2 supplementary material).

Twenty four farms out of 131 sampled had at least 1 animal positive (apparent prevalence 18.3%) (Table 3). The adjusted farm-level prevalence was 11.5% (95% credible interval 8.4%-16.0%) after adjusting for the number of pigs tested per farm and the imperfect sensitivity and specificity of the test; the credible interval refers to the sample estimate rather than a population estimate. The between-farm variance was 21.38, giving an intra-farm correlation of 0.99.

Seventy three farms (55.7%) returned a completed questionnaire. The median number of pigs in the farm at the time of sampling was 220 (1st and 3rd quartiles 31 and 2217 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 to have access to the farm (Table 4)

Out of those farms that returned a completed questionnaire (n=73), only two were deemed positive using a cut-off of ≥90% probability of having at least one infected animal; four farms were deemed positive using ≥80% cut-off and five farms using a cut-off of ≥50% (Figure 2). There were no statistically significant associations ($p \leq 0.05$) between farm status and any of the putative risk or protective factors explored (Table 4). This could be due to the lack of statistical power given the small number of positive farms (16% and 28% power of identifying a risk factor with OR≥2.5 and ≥3.5 respectively with 5 positive farms). Fifteen farms were deemed positive considering a lower cut-off: ≥10% probability of having at least one true positive, increasing the power to 30% (for OR≥2.5) and 50% (for OR≥3.5). Three farm characteristics were statistically significant from the univariate analysis; 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). These 3 variables exhibited strong collinearity (p<0.1) and therefore, the three univariate models were kept. Overall 17 (23.3%) of the farms had the three characteristics (small herds, outdoor access and allowed cats have access to pigs' fed), of which 7 farms (41.2%) were positive (≥10% probability).

Discussion

A low proportion of pigs tested positive in the current study (3.6%) with the majority of these having a low MAT titre. Some of the animals tested could have been sows or boars which may have increased the number of animals that tested positive. This suggests a low level of *T. gondii* infection in the farms studied, most of which are likely to send to slaughter batches composed of 100% uninfected pigs. Crucially, positive pigs came from a small number of farms (24 farms out of 131) and a very high intra-farm correlation was found, suggesting that the risk of *T. gondii* infection in pigs is largely driven by farm-level factors. In a previous study in the UK, 7.4% of pigs tested positive for *T. gondii* antibodies (Powell et al., 2016). Although important geographical overlap exists between studies, our study only included farms in England where 82% of the UK pig production is located (PHWC, 2015). The results are not directly comparable given the differences of study design and the test used.

Although the five collaborating slaughterhouses reflect the diversity of abattoirs in the country in terms of throughput, specialisation and type of farms (PHWC, 2015), voluntary participation of slaughterhouses and farms is a limitation of this study. However, one of the collaborating abattoirs is among the few in the country that slaughters finishing pigs only and has one of the highest throughputs. The remaining four slaughterhouses handle other species and two of them also slaughter boars and sows. Similarly, the farms in the study reflect the variability of pig production in England (PHWC, 2015).

Studies comparing the sensitivity and specificity of MAT and ELISA in naturally infected pigs, are scarce and results are contradictory (Dubey et al., 1995; Gamble et al., 2005). Variation of test results could be due to the *T. gondii* strain and time elapsed between infection and sampling (Dubey et al., 1997). Antibodies are detected by MAT 3 weeks post infection, peaking at week 6 and then decreasing but maintained permanently. Titres ≥1:320 are indicative of recent infection (Dubey et al., 1996). In this study a higher number of samples were classified as positive using MAT ($p<0.001$), which is aligned with results elsewhere (Steinparzer et al., 2015). MAT has been shown to have better precision and accuracy under experimental conditions, but it is time consuming, expensive and not commercially available. Conversely, ELISA is cheap, easy to conduct and commercially available, yet its accuracy is low. For surveillance proposes, ELISA could be used as a routine screening test, while MAT should be the test of preference if regional or national farm-level prevalence estimates are required.

Once adjusted for the number of animals tested per batch and the sensitivity and specificity of MAT, the farm-level prevalence was 11.5% (95% credible interval 8.4%-16.0%). 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 level of *T. gondii* infection appears to be lower than that reported by studies in Germany (69.1%) (Damriyasa et al., 2004), Italy (42.3%) (Villari et al., 2009), Spain (85.0%) Greece (26.2%) (Papatsiros et al., 2016) and Austria (23.3%) (Steinparzer et al., 2015). It is important to note that prevalence estimates reported in these studies were not adjusted for test sensitivity and specificity and the criteria for classification of positive farms varied.

Regional differences within some European countries have been reported. Farms located in regions with high temperatures and moderate rainfall in Spain had higher risk of infection than those located in regions below or above the average rain fall, and a similar pattern was reported outside Europe (Alvarado-Esquivel et al., 2014; Alvarado-Esquivel et al., 2015). Comparisons between areas on the basis of climatic conditions should be made with caution as there are likely to be other potential confounding effects, such as farm characteristics or management practices. However, it has been hypothesised that survival of oocysts might increase with humidity, while sporulation time might be shortened with higher temperatures (Dubey, 2010; Opsteegh et al., 2016). Although further studies are needed to explore the role of climatic conditions on the survival of *T. gondii* oocysts, English climatic conditions could potentially limit oocyst survival and therefore reduce the level of exposure and infection in pigs, compared to other climates.

Smaller herds (≤ 200 pigs) had a higher risk of infection (RR=3.0; p=0.02) which is in accordance with studies elsewhere (Villari et al., 2009; Zimmerman et al., 1990). Herd size is often related to other management practices and should not be considered as an isolated factor. In this study, farms with smaller herds were more likely to keep other livestock species, have a continuous cycle, allow outdoor access to pigs and have an open food storage.

Having outdoor access, presence of cats in the farm and feed stored with the possibility for contamination with cats' faeces, have been previously reported as risk factors for *T. gondii* infection (Assadi-Rad et al., 1995; Garcia-Bocanegra et al., 2010a; Garcia-Bocanegra et al., 2010b; Gebreyes et al., 2008; Guo et al., 2016; Kijlstra et al., 2004; Klun et al., 2006; Ortega-Pacheco et al., 2013; Tao et al., 2011; Weigel et al., 1995). In our study the relative risk of infection was higher on those farms where pigs had outdoor access at any production stage (RR=3.0; p=0.04). Keeping cats in the farm or cats from outside being able to access the farm were not significantly associated with *T. gondii* infection. However, cats having direct access to pigs' feed increased the risk of infection 2.6 fold and was significant (p=0.04) when a 10% cut-off was considered. Recommendations to farmers should emphasise the importance of ensuring cats do not have access to pigs' feed. 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. At EU level, requirements for controlled housing (Anonymous, 2015) could be amended to include mandatory feed storage in closed silos or containers impenetrable to cats, in order to distinguish between low and high biosecurity herds for *T. gondii*.

The true incidence of human toxoplasmosis in England is unknown as a result of underreporting; an enhanced surveillance programme in England and Wales introduced in 2008 (Halsby et al., 2014) identified 1824 confirmed cases during its first five years, with over a third of them coming from the London area. A previous study had reported a sero-prevalence of 17% among pregnant women in London, with African, Afro-Caribbean, Middle Eastern and mixed race ethnic origins and consumption of undercooked meat as the main risk factors (Flatt and Shetty, 2013). Lower sero-prevalence (9.9%) was reported in studies conducted in Northern England (Zadik et al., 1995) and Southern England (7.7%) (Allain et al., 1998) fifteen years previously. Both studies tested women during the antenatal screening, but risk factors were not reported.

The foodborne route has been considered as the most important route for human *T. gondii* infection in a recent WHO expert elicitation (WHO, 2015). Furthermore, consumption of undercooked meat (pork, beef and lamb) has repeatedly been found as a risk factor for *T. gondii* infection (Baril et al., 1999; Bobic et al., 2007; Cook et al., 2000; Flatt and Shetty, 2012; Jones et al., 2009; Kapperud et al., 1996), however the type of meat reported varies across countries. Ascertainment of the relative contribution of pork and other animal products to the risk of human *T. gondii* infection and of the effect of farm-level measures warrants a formal risk assessment in which risk mitigation measures along different stages of meat production chain are assessed by probabilistic risk modelling.

Conclusions

This study provides an approximation to the level of *T. gondii* infection in pigs raised in commercial farms in England using a novel method for prevalence estimation. It also investigates farm characteristics and management practices which may increase the risk of pigs becoming infected. Most of the batches included in this study were likely to contain 100% of uninfected pigs, with a small number of batches accounting for a large proportion of the positive pigs, which indicates that the risk of *T. gondii* infection is largely driven by farm-level factors. At pre-harvest level, mitigation of the risk of exposure to toxoplasmosis via consumption of pork should target farms with outdoor access and/or open feed storage. The study fills some of the data gaps previously identified by the UK Food Standard Agency (AMCSF, 2012) and provides inputs that could be used to populate probabilistic assessments of human foodborne exposure.

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Table 1. Variables considered in the standardised questionnaire to assess potential risk factors for *T. gondii* status in commercial pigs in England. Information collected between January and July 2015 (n=73)

Variable description and question asked in the questionnaire	Categories / options provided in the questionnaire	Variable re-grouped for analysis	
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 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	Have outdoor access at any production stage Yes No outdoor / indoor / part outdoor part part indoor	
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 held 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 STORAGE			
Where is the animal feed stored? Tick all that apply	Open silo Open storage Closed silo Closed 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)
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?*	-Yes, cats definitely come into direct contact with pigs / pigs' food / pigs' drinking water	Possible
CATS – CONTACT WITH PIGS' FOOD *Is it possible that cats come into contact with pigs' food?*	-Yes, it is very likely that cats come into contact with pigs/ pigs' food / pigs' drinking water -Not sure	
CATS – CONTACT WITH PIGS' DRINKING WATER *Is it possible that cats come into contact with pigs' drinking water?*	-No, cats cannot come into contact with pigs/ pig's food / pigs' drinking water	No possible
DE-WORMING *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

Table 2. MAT titres and ELISA results for serum samples tested for *T. gondii* (n=492). Samples collected between January and July 2015 from commercial pigs in England. Results in this table are not adjusted for the Sensitivity and Specificity of the test

MAT Status	Titre	ELISA *Positive*	ELISA *Inconclusive*	ELISA *Negative*	TOTAL
Positive	1:25	2	2	6	10
	1:50	8	5	11	24
	*1:100	5	1	5	11
	1:200	7	1	6	14
	1:400	5	0	1	6
	1:800	3	1	0	4
	1:1600	2	2	0	4
	*1:3200	0	0	0	0
	Total	**32 (4.9%)**	**12 (2.4%)**	**29 (5.9%)**	**73**
Suspicious	*1:1	1	0	31	32
	1:3	0	3	21	24
	1:6	1	2	13	16
	1:10	0	4	3	7
	Total	**2 (0.41%)**	**9 (1.8%)**	**68 (13.8%)**	**79**
Negative	**0**	**3 (0.61%)**	**3 (0.61%)**	**334 (67.9%)**	

*There was no serum left for three serum sample to be tested by ELISA – one sample with titre 1:10; one sample with titre 1:100 and one sample with titre 1:3200.

Table 3. Apparent batch-level prevalence for *T. gondii* in commercial pigs in England. Serum samples tested by MAT. Samples collected between January and July 2015 (n=131).

Apparent batch-level prevalence *	Number of farms	Herd size Median (1st – 3rd quartile)
0%	107	260 (32 - 2624)†
0.1 – 10%	11	
10.1 – 20%	4	
20.1 – 30%	1	
30.1 – 40%	2	
40.1 – 50%	1	
50.1 – 60%	1	66 (11 - 960)‡
60.1 – 70%	1	
70.1 – 80%	1	
80.1 – 90%	0	
90.1 – 100%	2	

*Results in this table are not adjusted for the number of pigs tested per batch/farm and MAT sensitivity and specificity. The number of animals included in a batch ranged from 1 to 235 pigs

‡Nine out of the 13 farms with >10% apparent within-herd prevalence returned a completed questionnaire. In 5 out of 13 farms (55.6%) pigs had outdoor access and in 5 farms (55.6%) cats had access to pigs' food.

†Sixty four out of the 118 farms with ≤10% apparent within-herd prevalence returned a completed questionnaire. In 30 out of 64 farms (46.9%) pigs had outdoor access and in 22 farms (34.4%) cats had access to pigs' food.

Table 4. Distribution of potential risk factors for *T. gondii* positive and negative pig farms in England following univariate analysis.

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 (20.0)		
• Small herds (1-200 pigs)	34 (50.0)	2 (40.0)	1	1.2	25 (43.1)	12 (80.0)	**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 (80.0)		
• No	58 (85.3)	3 (60.0)	0.18	3.4	9 (15.5)	3 (20.0)	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 (20.0)		
• Possible	41 (60.3)	4 (80.0)	0.64	2.5	33 (56.9)	12 (80.0)	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 (40.0)		
• Possible	24 (35.3)	3 (60.0)	0.35	2.6	18 (31.0)	9 (60.0)	**0.04**	2.6
Cats can get in contact with pigs' drinking water								
• No	44 (64.7)	3 (60.0)			40 (69.0)	7 (46.7)		
• Possible	24 (35.3)	2 (40.0)	1	1.2	18 (31.0)	8 (53.3)	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

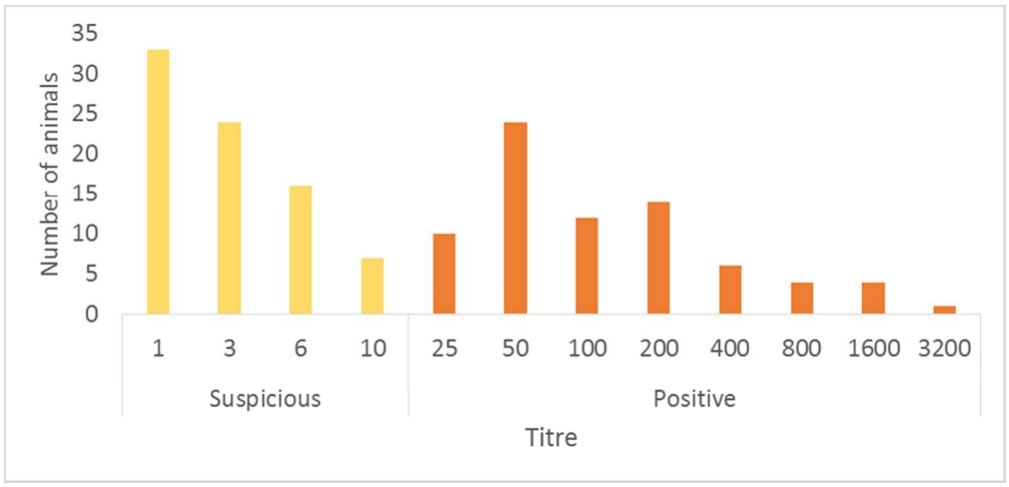

Figure 1. Number of suspicious (titre between 1:1 and 1:10) and positive (titre ≥1:25) pigs in England to T. gondii by MAT in each titre band. Samples collected between January and July 2015. Results in this figure are not adjusted for the sensitivity and specificity of the test.

Figure 1
160x76mm (150 x 150 DPI)

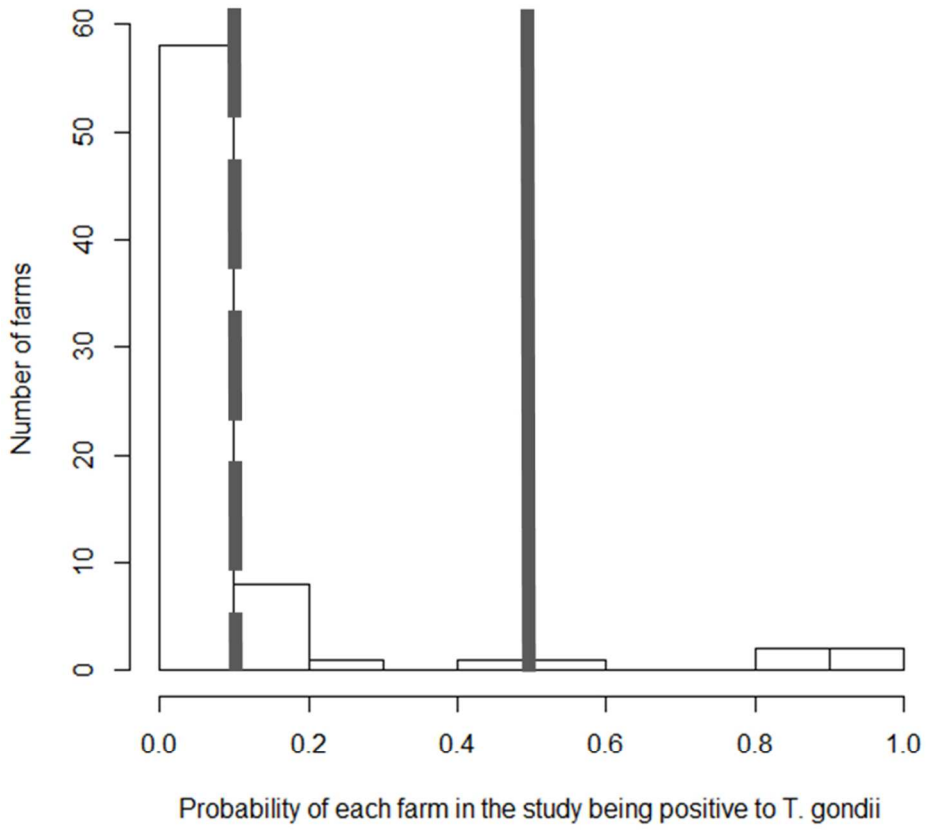

Figure 2. Frequency distribution of the probability of each English pig farm in the study being positive to T. gondii after adjusting for test sensitivity and specificity and proportion of animals sampled in each batch. Cut-off used to consider farms positive or negative are illustrated with a dashed line (≥10%) and a solid line (≥50%).
Figure 2
160x154mm (150 x 150 DPI)

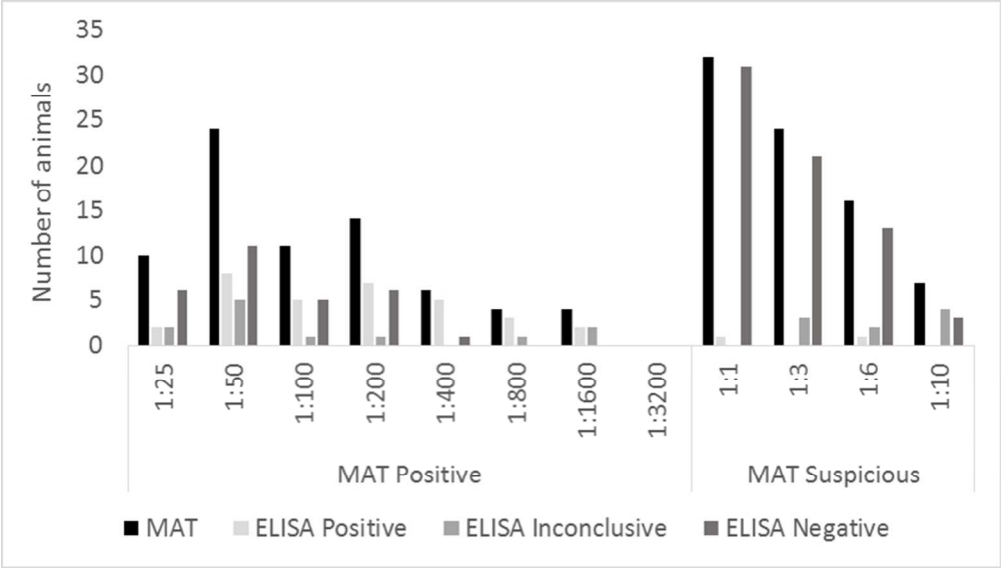

Figure S1. MAT titres and ELISA results for serum samples tested for T. gondii (n=492). Samples collected between January and July 2015 from commercial pigs in England. Results in this figure are not adjusted for the Sensitivity and Specificity of the test

Figure S1
142x81mm (150 x 150 DPI)

Table S2.1 Number of farms deemed positive to *T. gondii* after adjusting for MAT and ELISA sensitivity and specificity and proportion of pigs sampled in each batch. A farm was considered positive if the probability of having at least one pig positive was ≥50%.

MAT	ELISA			
	Positive	Negative	**Total**	**P value†**
Positive	6	2	**8**	
Negative	4	118	**122**	
Total	**10**	**120**	**130**	0.41

† McNemar's Chi-squared test

Table S2.2 Number of farms deemed positive to *T. gondii* after adjusting for MAT and ELISA sensitivity and specificity and proportion of pigs sampled in each batch. A farm was considered positive if the probability of having at least one pig positive was ≥10%

MAT	ELISA			
	Positive	Negative	**Total**	**P value†**
Positive	14	11	**25**	
Negative	3	102	**105**	
Total	**17**	**113**	**130**	0.03

† McNemar's Chi-squared test

Figures captions

Figure 1. Number of suspicious (titre between 1:1 and 1:10) and positive (titre ≥1:25) pigs in England to *T. gondii* by MAT 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.

Figure 2. Frequency distribution of the probability of each English pig farm in the study being positive to *T. gondii* 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 a dashed line (≥10%) and a solid line (≥50%).

Supplementary material

Figure S1. MAT titres and ELISA results for serum samples tested for *T. gondii* (n=492). Samples collected between January and July 2015 from commercial pigs in England. Results in this figure are not adjusted for the Sensitivity and Specificity of the test