EFSA EXTERNAL SCIENTIFIC REPORT

Experimental studies on *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report[1]

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ABSTRACT

After extensive review of the literature, experimental studies were performed in the main livestock species (cattle, small ruminants, pigs, horses and poultry) to address the main knowledge gaps considering (1) the relationship between detection of antibodies and presence of *T. gondii*, (2) the anatomical distribution of tissue cysts, and (3) on-farm risk factors for infection. In slaughterhouse studies in cattle and horses, a lack of concordance between detection of antibodies by MAT and detection of *T. gondii* in tissues by mouse bioassay or MC-PCR was demonstrated. Positive mouse bioassay results were obtained in cattle and horses, indicating a potential infection risk for consumers.

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[1] Question No EFSA-Q-2013-00207.

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Suggested citation: Opsteegh M, Schares G, Blaga R and van der Giessen J on behalf of the consortium, 2016. Experimental studies of of *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01) Final report. EFSA supporting publication 2016:EN-995, 161 pp.

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In naturally infected pigs and chickens the agreement depended on assays and matrices/analytes used, but was considered fair to substantial. Calves, sheep, pigs, chickens and turkeys were experimentally infected with *T. gondii*. In calves various tissues were tested and *T. gondii* was recovered, but no clear predilection sites were identified. There was also little variation in parasite load between the different edible tissues (i.e. skeletal muscles) from pigs and sheep. Brain and heart were clearly identified as predilection sites in chickens and turkeys, with higher parasite loads compared to breast, thigh and drumstick muscle. The observation that drumstick, an edible tissue, tested positive in 25% (8/32) of the bioassay positive organic laying hens, indicated a potential risk for consumers. The seroprevalence of *T. gondii* in Dutch indoor-housed dairy goats appears to be relatively low and was associated with the presence of cats at the farm. In the UK, the exposure of pigs to *T. gondii* appears to be low, and infected pigs were present at a limited number of farms. Laying hens with outdoor access were more often infected at small backyard farms compared to large organic farms in Germany. Vaccination with S48 strain *T. gondii* was shown to reduce tissue cyst formation in vaccination and challenge studies in sheep and pigs.

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KEY WORDS

Toxoplasma gondii, experimental studies, cattle, small ruminants, horses, pigs, poultry

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SUMMARY

Toxoplasma (T.) gondii, an intracellular coccidian parasite, is one of the most successful parasites worldwide. Felids are definitive hosts of this parasite, whose sexual reproduction takes place in the intestinal tract of this mammal resulting in shedding of oocysts. Virtually all warm blooded animals can carry tissue cysts and act as intermediate hosts. Humans, as aberrant intermediate hosts, become infected with *T. gondii* through ingestion of tissue cysts in raw or undercooked meat or oocycts from the environment. In women with primary infection during pregnancy, toxoplasmosis can result in abortion or cause chorioretinitis and various central nervous system abnormalities in the fetus. Furthermore, *T. gondii* can cause ocular toxoplasmosis after acquired infection in immunocompetent individuals and can lead to severe disease in immunocompromised individuals. Based on the disease burden (expressed in Quality or Disability Adjusted Life Years), *T. gondii* is one of the most important foodborne pathogens warranting the implementation of intervention measures. Meat appears to be a major source of *T. gondii* infections in Europe, therefore it is important to have an indication on the prevalence of infectious tissue cysts in the main livestock species.

The overall goal of the project was to gain information and knowledge on the presence and infectivity of *T. gondii* cysts in meat and other edible tissues in the main meat-producing animals and its relationship with *T. gondii* seroprevalence in animals. In this report, we describe the results of the experimental studies in the main livestock species (pigs, cattle, sheep, goats, chickens, turkeys and horses). The main questions we studied were (i) the relationship between indirect detection methods and direct detection methods for presence and levels of infective cysts in meat and other edible tissues of slaughtered cattle in four countries (the Netherlands (NL), Italy (IT), the United Kingdom (UK) and Romania (RO); in slaughtered horses in two countries (France (FR) and Serbia (RS)); in slaughtered pigs in FR; in organic poultry in Germany (DE); (ii) the anatomical distribution of the cysts in meat and other edible tissues after experimental infection in calves, sheep, pigs, chickens and turkeys; (iii) risk factors for *T. gondii* infection in pigs in the UK, in indoor kept dairy goats in NL, and in organic laying hens with outdoor access in DE; (iv) the presence of tissue cysts after vaccination in pigs and sheep.

Relationship between direct and indirect detection methods in cattle. To be able to study the correlation between serological results and presence of tissue cysts in cattle, serum, liver (identified as predilection site in literature review) and diaphragm samples of veal and adult cattle were obtained from slaughterhouses in the NL, IT, RO and UK. Liver samples were analysed by mouse bioassay (BA) and quantitative PCR (qPCR) on liver digest. In addition, a selection of diaphragms from negative cattle and all diaphragms of bioassay, digest PCR or serologically positive cattle were analysed by magnetic capture qPCR (MC-PCR). Serum samples were tested for anti-*T. gondii* IgG with MAT. To analyse the concordance between direct and indirect tests, kappa values were determined. A total of 402 cattle were analysed. Overall, 13 animals were considered direct detection-positive, and for 6 out of 385 cattle this conclusion is based on BA (1.6%), indicating the presence of viable tissue cysts and thus a potential risk for consumers. For 15 cattle, no BA results were obtained as the mice died. The six positive BAs concerned two mice from NL (corresponding to two cattle) and 5 mice from IT (corresponding to 4 cattle), which were positive in PCR on the brain, but negative in MAT. All seven cattle positive in MC-PCR on the diaphragm originated from RO. These results demonstrate a lack of concordance between the identification of viable tissue cysts in liver and the detection of *T. gondii* DNA in the edible tissue. In addition, the probability to directly detect *T. gondii* in seropositive and seronegative cattle is similar, demonstrating that serological testing by MAT does not provide information about the presence of *T. gondii* in cattle and does not provide an indication of the risk for consumers.

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Anatomical distribution in calves. To determine the dissemination of *T. gondii* tissue cysts to different tissues, organs and meat cuts in cattle, six Holstein Friesians calves (*Bos taurus*), aged 6 weeks, were orally inoculated with $1x10^6$ *T. gondii* oocysts of the M4 strain. Tissues were collected and tested in two pools by BA, qPCR on tissue digests and MC-PCR. Calves did not develop clinical symptoms, but all animals developed an antibody response detactable by MAT between D14 and D21. Despite all calves showed seropositivity, tissue cysts could not be detected in all animals. Most of the direct detection was performed on pooled tissues. *T. gondii* was shown to disseminate to various tissues in calves after oral inoculation with 10^6 oocysts including meat cuts, and both viable parasites and DNA were detected with good agreement between BA and MC-PCR results. But no clear predilection sites could be identified.

Risk factors in dairy goats. Controlled housing is known to reduce exposure to *T. gondii* in pigs and poultry, but little is known concerning the seroprevalence of *T. gondii* in indoor-kept dairy goats. To determine the seroprevalence and to identify risk factors associated with *T. gondii* seroprevalence in Dutch indoor-housed dairy goats, a commercial indirect ELISA was used to test 1664 goat sera from 52 farms. Seroprevalence of *T. gondii* infection was estimated at 13.3%, and 61.5% of farms had at least one seropositive goat present. Number of cats was a significant predictor for the seroprevalence of goats in the farm. This seroprevalence was much lower than previously reported in goats (47%) and adult sheep (48.1%) in NL, but is higher than reported for indoor kept pigs and poultry, probably because goat housing is less confined than pig or poultry housing (with e.g. natural ventilation and bedding, silage and roughage introduced). Since the overall animal level seroprevalence was 13.3% and a positive relationship between detection of antibodies against *T. gondii* and presence of tissue cysts in meat has been reported in literature, goats could be a source of *T. gondii* infection for humans. Limiting the presence of cats at the goat farms is expected to reduce the prevalence of *T. gondii* infected goats.

Relationship between direct and indirect detection methods in pigs. In FR, the concordance between serology, bioassay and PCR-based detection in pigs was assessed using samples collected in a previously conducted nationwide study. For 160 selected pig samples, MAT results on three different matrices (sera, cardiac fluid and diaphragm fluid) were compared with the direct detection results (BA, qPCR and MC-PCR). Out of the three different matrices, the MAT results for cardiac fluid gave the highest concordance with direct detection. Therefore, only the MAT results on cardiac fluid were considered for further comparison with direct detection methods, and concordances of 0.66 (BA), 0.46 (PCR on digest), 0.23 (MC-PCR) and 0.41 (any direct detection) were found. Recovery by direct detection was significantly higher in seropositive than seronegative animals. Detection in seronegatives was higher than expected by PCR on heart digest (23.9%) and MC-PCR on diaphragm (10.2%), but comparable to literature based on mouse bioassay results (6.0%). The isolation of a *T. gondii* strain from a seronegative pig indicates the potential risk for human infections from MAT negative pigs.

Vaccination in sheep and pigs. Vaccination and challenge experiments had previously been peformed and published (Katzer et al., 2014; Burrells et al., 2015). In the present project, tissues were additionally tested by MC-PCR to analyse the effect of vaccination on parasite load. In line with published results, vaccination was shown to reduce tissue cysts formation and could therefore contribute to reducing transmission of *T. gondii* to humans via consumption of infected meat. In the unvaccinated but challenged control groups, MC-PCR results indicate that there is little variation in parasite load between the different skeletal muscles in sheep and pigs.

Risk factors in pigs. In the UK, limited information is available about the seroprevalence and risk factors in pigs. In total 2071 pigs from 131 farms were analysed by MAT. Antibodies (IgG) against *T. gondii* were found in 75 pigs (3.6%). Twenty-four farms had at least 1 animal positive (apparent

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prevalence 18.3%). There was no statistically significant association between positive farms and any of the putative risk or protective factors explored, probably due to a lack of power resulting from the small number of positive herds.

Relationship between direct and indirect detection methods in horses. Eating raw horse meat may expose consumers to *T. gondii*. The aim of the horse study was similar to the cattle study as described above: to assess the correlation between indirect detection (serology by MAT) and direct detection (mouse bioassay and PCR-based detection on tissue digests and magnetic capture qPCR (MC-PCR)) on a selected edible tissue. The study was performed in two countries, FR and RS. One-hundred eighty horse samples were collected in one slaughterhouse in FR and one-hundred four samples from two slaughterhouses in RS. The seroprevalence in RS (48.1%) was significantly higher than in FR (27.8%). Overall, in FR, 15 horses (8.2%) were reported as positive in at least one of the direct techniques, while 69 positive horses were identified in RS (66.3%). Although a real difference in prevalence is possible, practical issues with the qPCR procedure are considered a more plausible explanation. Therefore, only the microscopy results were taken into account for BA in RS. Overall, 33 animals were considered direct detection-positive (11.6%), and for 12 of them this conclusion is based on BA (4.2%), indicating the presence of viable tissue cysts. Similar to cattle, there is a poor concordance between the presence of antibodies against *T. gondii* and direct detection of the parasite. In RS, two *T. gondii* strains were isolated demonstrating the presence of infectious *T. gondii* in horses.

Relationship between direct and indirect detection methods in organic laying hens. Chickens and turkeys are known to be highly susceptible to *T. gondii* infection; however, industrialized husbandry has decreased *T. gondii* exposure for poultry. With the increasing demand for organic meat, the risk of human infection from organic poultry meat is potentially on the increase. In poultry, two studies were carried out. One field study focused on laying hens from organic farms with the aim to study the concordance between results from indirect tests (MAT, ELISA and IFAT) and the detection of *T. gondii* in heart and drumstick by direct detection methods (BA, PCR on digest and MC-PCR). In addition, a questionnaire was included in the study to analyse putative risk factors. From a total of 16 farms, 61 laying hens were selected for the further analyses. The results of the different direct detection methods agreed well with each other (Kappa-values ranging from 0.77 to 0.87). In general, on farms with a high seroprevalence in hens at the end of laying period, there was a high probability of identifying positive chickens by bioassay or MC-PCR. Kappa values characterizing the agreement between BA, digest PCR or MC-PCR and antibody detection in serum ranged from 0.55 to 0.77, with ELISA and IFAT generally showing higher concordance with direct detection than MAT. Results of our study suggest that serology is able to identify farms with *T. gondii* tissue cysts positive hens. In none of the large commercial organic farms isolation of viable *T. gondii* by BA was achieved. In univariate analysis, variables associated with small farms size (the use of cats for rodent control, low chicken density, presence of empty period and long cleaning interval) were associated with a >20% seroprevalence (ELISA) on the farm. This suggests that prevalence and parasite load in backyard hens is higher than in hens from large organic farms.

Experimental infections in poultry. To study the anatomical distribution of *T. gondii* in experimentally infected chickens and turkeys; brain, heart, breast muscle, thigh, and drumstick were tested by MC-PCR. In addition, the performance of MAT, IFAT and ELISA were evaluated. Inoculation using oocysts or tissue cysts was more effective in inducing a measurable serological response and tissue cyst formation than inoculation with tachyzoites. All serological methods for antibody detection in serum demonstrated 100% specificity and sensitivity ranged from 89.0% to 100.0%. *T. gondii* was detected by MC-PCR in 89.7% of chickens and 97.5% of turkeys inoculated with oocysts. MC-PCR results in both chicken and turkey suggest that there is at least 100-fold more parasite DNA in brain or heart than in breast, thigh or drumstick musculature. In addition, diagnostic characteristics of the serology were evaluated to identify chickens and turkeys which tested *T. gondii* positive by MC-PCR.

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Under experimental conditions, MC-PCR results suggest moderate to substantial agreement between the IFAT and ELISA results, independently of the analyte (serum, body fluids), but MAT shows a high proportion of false positives in body fluids; these false positive reactions were responsible for a low agreement between MAT and MC-PCR from breast, thigh or drumstick.

Conclusions

Concerning the first objective (relationship between indirect and direct detection methods) it was concluded that:

- MAT based detection of antibodies, and possibly serological screening in general, are not recommended as an indicator of the presence of viable *T. gondii* in cattle and horses. In these species, direct detection methods are preferred;
- The detection of antibodies appears to be useful to estimate the extent of viable *T. gondii* in pigs and poultry. However, viable *T. gondii* was also detected by mouse bioassay in <10% of seronegative pigs and chickens; therefore serological screening cannot be used to declare the meat of individual animals as *T. gondii* free. The ability to identify animals harboring *T. gondii* using an indirect detection method is influenced by the the assay and matrix/analyte used for antibody detection;
- No experimental studies were performed in small ruminants to analyse the concordance between direct and indirect tests, but based on the literature review the concordance in sheep and goats is expected to be similar to the concordance determined in pigs and poultry.

Concerning the second objective (anatomical distribution), it was concluded that:

- There is little variation in parasite load between different skeletal muscles in pigs and sheep. Clear predilection sites (brain, heart and lung) have been identified in MC-PCR based studies described in the literature;
- *T. gondii* was detected in several types of tissues of experimentally infected calves, but no clear predilection sites were identified;
- Brain and heart were identified as predilection sites in experimentally infected chickens and turkeys. Estimated parasite loads were 100 to 1000-fold higher in brain and heart than in breast, thigh or drumstick muscle of the same animals;
- Comparing recovery rates and parasite loads in the different species, is hampered by differences in study design and sampling. However, the results appear to confirm the lower susceptibility and limited parasite load in cattle as has been suggested by the literature;
- Inoculation route did not affect the anatomical distribution in the pig, chicken and turkey infection experiments.

Concerning the third objective (on-farm risk factors), it was concluded that:

- Indoor-housed dairy goats in the Netherlands are exposed to *T. gondii*, but the seroprevalence appears to be relatively low. The presence of cats was associated with increased on-farm seroprevalence;
- Exposure to *T. gondii* in pigs in the UK appears to be low and infected pigs are present at a small number of farms;
- For laying hens with outdoor access in Germany, the risk of *T. gondii* infection was higher in backyard systems or at small compared to large organic farms;
- Experimentally, vaccination with S48-strain *T. gondii* has been shown to reduce or prevent tissue cyst development in sheep and pigs.

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Recommendations

For the first objective (relationship between direct and indirect detection):

- With currently available serological methods, implementation of serological screening to identify high risk herds or animals is not considered useful for cattle and horses.
- For pigs, poultry and small ruminants serological screening can be used to identify high risk herds or animals. However, a negative result in an indirect test can not be used to declare that the meat is safe.
- In sheep vaccination status should be considered if serological testing would be implemented to identify high risk herds or animals.

For the second objective (anatomical distribution):

- Further experimental studies for the purpose of studying the anatomical distribution in cattle are not advised unless more sensitive methods will become available.
- Experimental infection in horses is needed to study anatomical distribution and identify preferred tissues for testing.
- Direct testing of a predilection site (brain and heart) in pigs, small ruminants and poultry will make most efficient use of resources to determine the prevalence of animals harbouring tissue cysts.

For the last objective (risk factors):

- Risk factors in cattle and horses studies should be based on a direct rather than on currently available indirect detection methods.
- Housing information is unlikely to be useful for risk classification in indoor kept goats.
- The possibility to reduce *T. gondii* transmission to consumers by vaccination of sheep and goats should be further evaluated.
- Intervention studies are needed to determine the effectiveness of preventing exposure to risk factors

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BACKGROUND AS PROVIDED BY EFSA

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii*, and it is one of the most widespread parasitic diseases throughout the world. *Toxoplasma* infection is estimated to be present in 50%-80% of the European human population.[2] Most cases (80-90%) are asymptomatic and the majority of the remainder have only mild, self-limiting symptoms. However, severe complications may occur in immunocompromised individuals and after congenital *Toxoplasma* infection in seronegative pregnant women. A recent editorial stressed the need for more careful assessment of the prevalence and the potential risk for food-borne human toxoplasmosis; especially due to the suspicion that the organism could also contribute to psychiatric disorders.[3]

The parasite only matures in domestic and wild cats, which are the definitive hosts. Nearly all warm-blooded animals can act as intermediate hosts, and seemingly all animals may be carriers of tissue cysts of this parasite. Human infection may be acquired through the consumption of undercooked meat or food/water contaminated with oocysts shed in cat faeces or from handling contaminated soil or cat litter trays.[2] A European multicentre case-control study published in 2000 estimated that between 30% and 63% of acute infection in pregnant women in various European cities were attributed to consumption of raw or undercooked meat and 6% to 17% to soil contact.[4] Overall, the relative source attribution (i.e. direct contact, environmental, water-borne, food-borne) and exposure pathways of toxoplasmosis to humans (general population) remains undetermined.

Based on seroprevalence data in UK livestock species, *Toxoplasma* infection is most common in sheep, pigs and wild game. Cattle appear to be relatively resistant to infection. *Toxoplasma* has also been found in a wide variety of meats. Based on the current evidence, it was concluded that beef and housed chicken appear less commonly infected than other meats.[2] In the EU, the highest proportion of samples positive for *Toxoplasma* or antibodies across all reporting Member States (MSs) was reported for sheep and goats.[2] It was estimated that 68%, 14%, 11% and 7% of the meat-borne infections in the Netherlands are due to beef, sheep, pork and mixed meat products, respectively.[5] In the USA[6] rankings of 168 food-pathogen combinations were developed. Considering the cost of illness, *T. gondii* ranked in the top-50 for six foods: pork (top 2), beef (top 8), produce, poultry, dairy products and deli meats.

EFSA published a Scientific Opinion of the BIOHAZ Panel on *Toxoplasma* in 2007.[7] In 2010, EFSA received a mandate from the European Commission on the modernisation of meat inspection from various species in the EU. Among the main objectives of these opinions a key one is to identify and rank the meat-borne hazards so to identify the most relevant ones for each animal species. The Scientific Opinions on the public health hazards as related to inspection of meat of swine[8] and poultry[9] have been published. In the Opinion on meat inspection of swine, the BIOHAZ Panel concluded that,

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2 Available at http://www.efsa.europa.eu/de/efsajournal/doc/2597.pdf.

3 Anonymous, 2012. *Toxoplasma gondii*: an unknown quantity. The Lancet Infectious Diseases (editorial), 12: 737.

4 Cook et al., 2000. Sources of toxoplasma infection in pregnant women: European multicentre case-control study. British Medical Journal, 321: 142-147.

5 Opsteegh et al., 2011. A quantitative microbial risk assessment for meatborne *Toxoplasma gondii* infection in The Netherlands. International Journal of Food Microbiology 150: 103–114.

6 Batz et al., 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. Journal of Food Protection, 75 (7): 1278-1291.

7 Available at http://www.efsa.europa.eu/en/efsajournal/pub/583.htm.

8 Available at http://www.efsa.europa.eu/en/efsajournal/doc/2351.pdf.

9 Available at http://www.efsa.europa.eu/en/efsajournal/doc/2741.pdf.

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using risk ranking, *Salmonella* spp. are considered of high relevance and *T. gondii* of medium relevance. These assessments were based on their prevalence in/on chilled carcases, incidence and severity of disease in humans, and source attribution of hazards to pork. It was indicated that many data for hazard ranking were insufficient, and expert judgement was used instead. Data gaps were particularly evident in the case of *Toxoplasma*, for example regarding source attribution of human toxoplasmosis[2].

One of the main difficulties associated with the *Toxoplasma* ranking in the opinions was that most of the available data relating to the occurrence of *Toxoplasma* in animals were obtained by serological methods. Such evidence confirms that the animal has been exposed to the agent but does not inform whether the meat contains viable cysts at slaughter i.e. on the *Toxoplasma* risk posed by the meat. On the other hand, much fewer data have been reported on testing of the meat for the presence of *Toxoplasma*; and those data were generated mainly by PCR methodology. However, PCR-based positive results of meat testing also do not indicate the level of risk posed by the meat. Furthermore, it is unclear whether, and to which extent, the positive serological findings in the animals, and the positive findings based on the meat testing are correlated to *Toxoplasma* infectivity.

TERMS OF REFERENCE AS PROVIDED BY EFSA

The overall goal of the project resulting from the present call for proposals is to gain information on the presence and infectivity of *Toxoplasma* cysts in meat and other edible tissues in main meat-producing animals and its relationship with *Toxoplasma* seroprevalence in animals.

The major objectives of the project resulting from this call for proposals are as follows:

- To carry out an extensive literature search[10] and review of available data on the relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues; determine risk factors for *T. gondii* infection in the main livestock species; select methods for detecting presence and infectivity of *T. gondii* cysts; and determine the anatomical distribution of the cysts in meat and other edible tissues.

- To perform experimental studies in meat-producing livestock species in the EU in order to collect relevant data to assess the relationship between *Toxoplasma* seroprevalence and presence and levels of infective cysts; to determine the anatomical distribution of the cysts in their meat and other edible tissues; and to identify on-farm risk factors for *T. gondii* infection in each animal species.

This grant was awarded by EFSA to: Beneficiary Dr. J.W.B. van der Giessen, coordinator. RIVM, the Netherlands

Grant title: Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat

Grant number: GP/EFSA/BIOHAZ/2013/01

[10] Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01), An extensive literature review.

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INTRODUCTION AND OBJECTIVES

Toxoplasma gondii is an intracellular coccidian parasite and one of the most successful parasites worldwide. Sexual reproduction resulting in shedding of oocysts occurs only in felids (definitive hosts), but virtually all warm-blooded animals can carry tissue cysts and act as intermediate hosts. Humans, as intermediate hosts, become infected with *T. gondii* through ingestion of oocysts (e.g. when handling soil or cat litter, via water or on unwashed vegetables) or tissue cysts in raw or undercooked meat. If a woman becomes infected for the first time during pregnancy, *T. gondii* is transmitted to the fetus in approximately 30% of the occasions (Thiebaut et al., 2007). This can result in abortion or a baby born with central nervous system abnormalities, chorioretinitis, unspecific signs, or without symptoms. *T. gondii* is also an important cause of disease in immune-compromised individuals, and was a major cause of death in AIDS-patients before the introduction of highly-active retroviral therapy (Luft and Remington, 1992). Postnatal *T. gondii* infection has long been perceived as harmless, but is now recognized as an important cause of chorioretinitis for immune-competent individuals (Gilbert and Stanford, 2000). Based on the disease burden (expressed in Quality or Disability Adjusted Life Years), *T. gondii* ranked second out of 14 foodborne pathogens in the USA (Batz et al., 2012), and first in the Netherlands (Havelaar et al., 2012), warranting the implementation of intervention measures. Meat appears to be a major source of *T. gondii* infections in Europe, as in an European multi-center case control study 30 to 63% of infections in pregnant women were attributed to meat, whereas 6 to 17% were most likely soil borne (Cook et al., 2000). To gain more insight into the role of meat as a source of human infection with *T. gondii*, it is important to have an indication on the prevalence of infectious tissue cysts in the main livestock species. This is generally studied using serological assays, and the seroprevalence of *T. gondii* infection in livestock raised outdoors (e.g. sheep, cattle) is generally high, whereas the seroprevalence in livestock raised indoors (e.g. indoor housed pigs and poultry) is low (Kijlstra and Jongert, 2008). However, the detection of antibodies to *T. gondii* in animals does not necessarily provide a good indication of the presence of infectious tissue cysts and the risk of human infection. Although theoretically there should be a strong correlation, as both antibodies and tissue cysts are assumed to persist life-long in sheep (Dubey, 2009b) and pigs (Dubey, 2009a), studies comparing indirect and direct detection methods are limited. Especially in cattle, detection of antibodies is common whereas successful isolations by bioassay are very limited (Dubey, 1986). In addition, *T. gondii* DNA has been detected in seronegative cattle (Opsteegh et al., 2011). Information on the prevalence of infective tissue cysts by species as well as by tissue within a species is urgently needed to assess the relative importance of different types of meat in human infection, e.g. by quantitative microbial risk assessment. In addition, the correlation between infective tissue cysts and seropositivity will give an indication of the usefulness of serological screening to classify livestock into different *T. gondii* risk categories, or to evaluate on-farm risk factors for *T. gondii* infections to inform potential intervention measures.

To reduce the risk of humans to become infected with *T. gondii* either congenitally or post-natally it is important to know potential risk factors associated with the infection of farm animals with the parasite. This knowledge is essential for the future implementation of Hazard Analysis and Critical Control Points (HACCP) allowing the farmers to develop efficient and sustainable control measures against *T. gondii* infection for their farms (Kijlstra et al., 2009; Meerburg et al., 2006).

This project was carried out in two phases. The objective of the of the first phase of the project was to carry out an extensive literature search and review available data on *T. gondii* in meat of the main livestock species (e.g. pigs, ruminants, poultry, and solipeds) to provide information on: (1) the anatomical distribution of *Toxoplasma (T.) gondii* tissue cysts, (2) the performance of direct detection methods, (3) the relationship between detection of antibodies to *T. gondii* and presence of *T. gondii* tissue cysts, and (4) on-farm risk factors for *T. gondii* infection in the main livestock species. The

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results of the literature review were reported in the final 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'. Results from the literature review were taken into account to select and design the studies for the experimental phase of the project. For example, the information on the anatomical distribution was used to determine sampling sites and the conclusions on the performance of direct detection methods were used to select mouse bioassay, PCR on digest and MC-PCR as direct detection methods.

The overall objectives of the experimental studies on *T. gondii* in meat from the main livestock species (ruminants, pigs, horses and poultry) are provided below. The emphasis on different objectives and approaches varied by species, depending on existing knowledge gaps.

(i) assess the relationship between indirect detection methods (e.g. *T. gondii* seroprevalence) and direct detection methods for presence and levels of infective cysts in meat and other edible tissues (e.g. bio-assay).

- Slaughterhouse or farm studies were performed in naturally infected cattle, horses, pigs and organic hens.

(ii) evaluate the anatomical distribution of the cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals.

- Experimental infections were performed in calves, sheep, pigs, chickens and turkeys.

(iii) collect information to identify on-farm risk factors for *T. gondii* infection in each animal species.

- Risk-factor studies were performed in indoor-housed Dutch dairy goats, pigs in the UK, and hens with outdoor access in Germany.

- The effect of vaccination on tissue cyst formation was studied experimentally in sheep and pigs.

The experimental studies on *T. gondii* in meat were divided into four species-specific sections (ruminants, pigs, horses, poultry). These experimental studies were organised in work packages as outlined in the following section (Project organisation and management).

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PROJECT ORGANISATION AND MANAGEMENT

The project was coordinated by RIVM (project coordinator: Joke van der Giessen) positioned at Centre for Zoonoses and Environmental Microbiology (Z&O). Arie Havelaar, member of the EFSA Scientific Panel on Biological Hazards and working at RIVM (Z&O) was involved in the project to advise directly the coordinator and the consortium. The partners were: National Institute for Public Health and the Environment (RIVM) and Central Veterinary Institute (DLO-CVI), the Netherlands; National Veterinary School of Alfort (ENVA –JRU BIPAR) and French Agency for Food, Environmental and Occupational health and Safety (ANSES –USC EpiToxo), France; Friedrich Loeffler Institute (FLI) and University Leipzig, Germany; University of Agricultural Science and Veterinary Medicine Cluj-Napoca (UASVM CN), Romania; Instituto Superiori di Sanita (ISS), Italy; The Royal Veterinary College (RVC), The Food Standards Agency (FSA), and the Moredun Research Institute, UK; The University of Belgrade Institute for Medical Research (IMR), Serbia. RVC and Moredun were financed directly via the Food Standards Agency, thus the UK partners and subcontractor claimed no money from EFSA. In this way, a broad range of countries representing the Northwestern, Central, Eastern and Southern part of the EU were represented in this experienced consortium. Four members of the consortium were assigned work package (WP) leaders in order to facilitate direct communication with the coordinator for their specific WP tasks, to communicate within their WP with their scientific staff members and between the WP's. WP leaders organised the work in the particular topics and communicate further with the members of the work package. WP leaders were as follows: WP1, 4 and 9 Joke van der Giessen (RIVM, the Netherlands) also the coordinator; WP2 Marieke Opsteegh (RIVM, the Netherlands); WP3 Gereon Schares (FLI, Germany); WP5 Marieke Opsteegh (RIVM, the Netherlands); WP6 and WP7 Radu Blaga (ENVA, France); WP8 Gereon Schares (FLI, Germany). The other members of the consortium supported both partner leaders in WP2 and WP3 and their specific partner leader in the WP5-8. All members supported WP4 and WP9 partner leader (Fig.1).

In order to achieve the objectives of this call, the project has been divided into 9 WP's as shown in Figure 1. In addition to WP1 (Management and communication) and WP9 (Knowledge integration and project reporting), seven separate scientific work packages (WP2-8) were defined, which grouped the objectives of the projectThe framework of the project covered 2 main work areas: gathering of current information by a systematic review approach and evaluation of *T. gondii* in meat-producing livestock species in the EU by an experimental study approach.

WP2 and WP3 concerned the literature review and are reported separately. In WP4 the results of the literature review were used to design the studies of the experimental phase of the project. WP5 focussed on ruminants and included: the slaughterhouse study of veal and adult cattle in four countries, the anatomical distribution after experimental infections in calves, the risk factor study in indoor kept dairy goats and additional testing of sheep samples after vaccination and challenge experiments. The experiments and results are described in chapter 1. In WP6, the concordance between direct and indirect tests in slaughter pigs in France, the additional testing of pig samples after vaccination and challenge experiments, and seroprevalence and risk factors in slaughtered pigs in the UK were studied. These experiments and results are described in chapter 2. In WP7, the concordance between direct and indirect tests in horses in France and Serbia were studied. These experiments and results are described in chapter 3. In WP8, the concordance between various serologic tests and direct detection methods and risk factors in organic laying hens and the anatomical distribution after experimental infections in chicken and turkeys were studied. These experiments and their results are described in chapter 4.

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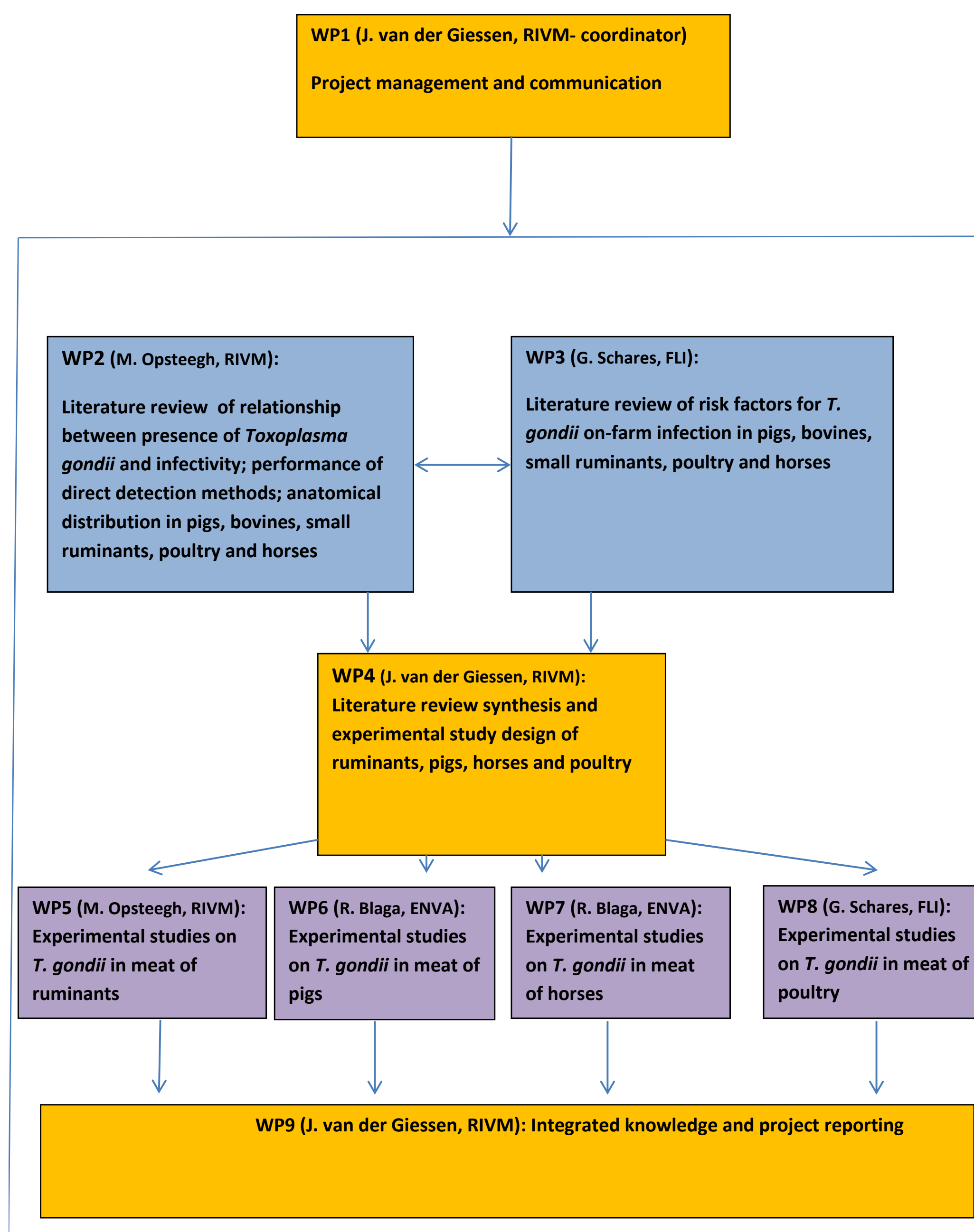

Figure 1: Project organisation and management

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1. Experimental studies in ruminants

Four studies were performed in ruminants and are described in the following sections. First, a slaughterhouse study was performed in four countries to study the concordance between detection of antibodies by MAT and the presence of parasites in cattle (1.1). Second, to study the anatomical distribution of tissue cysts, calves were experimentally inoculated with oocysts (1.2). Third, ovine tissues from a vaccination and challenge experiment in previously performed at Moredun were additionally tested by MC-PCR (1.3). Last, the risk factors for *T. gondii* infection in indoor-housed dairy goats were studied in the Netherlands (1.4).

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

1.1.1. Introduction

In cattle, the presence of *T. gondii* tissue cysts is debated and there appears to be a lack of correlation between the presence of antibodies and detection of tissue cysts. Although high seroprevalences have been reported, detection of *T. gondii* by bioassay is limited. Detection using PCR-based methods appears to be more common, and raises the question of infectivity. In addition, there is an indication that direct detection by PCR is more common in seronegative rather than seropositive cattle (Opsteegh et al., 2011). The aim of this study was to assess the concordance 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 study in four countries, namely the Netherlands (NL), the United Kingdom (UK), Romania (RO), and Italy (IT).

In cattle, a low prevalence of tissue cysts is expected. To be able to study the correlation between serological results and presence of tissue cysts, it is necessary to have a reasonable amount of bioassay or PCR positives. In general, *T. gondii* prevalence increases with age, however, in cattle there are studies that report a higher prevalence of *T. gondii* in younger than in older animals and it has been suggested that tissue cysts are not very persistent in cattle. For example, in Switzerland a higher prevalence of *T. gondii* was reported in calves (14/47) compared to cows (5/130), heifers (0/129) and bulls (0/100) as detected by PCR on one gram samples of the diaphragm (Berger-Schoch et al., 2011). Therefore, we aimed to sample equal numbers of calves and older cattle.

To have a reliable estimate of the concordance between serological results and the presence of tissue cysts a predilection site was sampled. A second tissue that is representative of edible tissues was sampled to give an indication of the distribution of tissue cysts in meat and thereby the risk for consumers. To decide on the tissues a literature review was performed on the anatomical distribution of tissue cysts in cattle. The results are presented in Table 1. From this table, it is clear that the small intestine and the liver are predilection sites for *T. gondii* in cattle and skirt steak, lymph nodes, thigh muscle and top round steak scored well based on a limited number of studies. Liver is easier to collect and better suitable for bioassay in mice as compared to small intestines. Therefore, liver was sampled as a predilection site. Diaphragm was sampled as a representative of edible tissue, since the average within study score and the fraction of positive studies are similar to those for the combined muscle and meat score (see Table 1).

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The following hypotheses were tested:

1. *T. gondii* can be found in cattle using direct detection methods (mouse bioassay, MC-PCR)

2. There is poor concordance between the results using direct and indirect (MAT) detection methods in cattle.

Table 1: *T. gondii* detection in 15 top-ranking bovine tissues, ranked by weighted (W) summed score

	Average within study score	**Number of studies**		**Fraction positive studies**		**Summed score**	
		Tissue tested	Positive results	UW[a]	W[b]	UW[a]	W[b]
muscle "skirt steak"*	0.67	1	1	1.00	0.06	1.67	0.73
unspecified lymph nodes	0.55	2	2	1.00	0.13	1.55	0.68
thigh muscle*	0.38	5	4	0.80	0.25	1.18	0.63
small intestine	0.31	7	5	0.71	0.31	1.03	0.63
liver	0.30	11	5	0.45	0.31	0.76	0.62
muscle "top round steak"*	0.50	1	1	1.00	0.06	1.50	0.56
muscle/meat combined	0.12	16	7	0.44	0.44	0.56	0.56
brain	0.15	15	5	0.33	0.31	0.48	0.46
blood	0.40	2	1	0.50	0.06	0.90	0.46
tongue	0.25	7	3	0.43	0.19	0.68	0.44
diaphragm	0.19	6	3	0.50	0.19	0.69	0.38
masseter muscle*	0.25	2	1	0.50	0.06	0.75	0.31
heart	0.12	15	3	0.20	0.19	0.32	0.30
unspecified muscle*	0.20	2	1	0.50	0.06	0.70	0.26
testicle	0.20	2	1	0.50	0.06	0.70	0.26

[a]UW: unweighted, [b]W: weighted
* These meat cuts and muscles were included in the "meat/muscle combined" category

1.1.2. Material and Methods

1.1.2.1. Sample collection

The sample size was set at 100 cattle per country to stay within the sampling and bioassay capacities in each country. To determine the sampling strategy an inventory per country was made showing the type, sex and age of animals that are being slaughtered (Table 2). Calves are defined as cattle less than 12 months old intended for slaughter as calves (Dec. 94/433/EC). Sampling equal numbers of calves and adult cattle is feasible in NL, IT and RO, but in the UK slaughtering of calves is very uncommon and the sampling target was set at 16 calf and 84 adult cattle samples.

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Table 2: Average number of cattle slaughtered in the Netherlands (NL), the United Kingdom (UK), Italy (IT), and Romania (RO) in 2013

	(Veal) Calves < 1 year (x 1000)	**Heifers & young bulls older than 12 months** (x 1000)	**Cows & adult bulls** (x 1000)	**Total** (x 1000)
NL[1]	1400 (73%)[$]	12 & 56 (3.5%)	500 & 56 (26%)	1900
UK	20 (3.3 %)	166 & 68 & 210 (73.7%)	138 (23%)	602
IT	547[a] (33%)	349[a] (22%)	731[a] (45%)	1627[a]
RO[2]	≤30 months = 56** (46%)		>30 months = 65** (54%)	139 (121**+18***)

[1] Data from StatLine: the electronic databank of Statistics Netherlands (statline.cbs.nl)
$: not all originate from the Netherlands, we intend to sample Dutch calves
[2] Data obtained in 15 May 2014 from National Sanitary Veterinary and Food Safety Authority from Romania
**ordinary slaughtering in 2013
***necessity and emergency slaughtering in 2013
[a]: all the animals were born, reared and slaughtered in Italy

In every country, sample collection was performed at slaughter. The number of slaughterhouses visited differed per country as presented in Table 3. Cattle were coded with a unique ID and it was ensured that matching samples were labelled correctly. A minimum of 4 ml of blood was collected in a 9 ml serum tube at bleeding or from the heart during evisceration. A minimum of 200 g of muscular part of the diaphragm and 400 g of liver was collected in separate seal bags. Sampling was limited to one animal per farm, and age, sex and breed were noted. Samples were kept and transported on ice or in the refrigerator as much as possible. The liver was processed for mouse bioassay the day after sample collection. Diaphragm samples were stored at -20°C and, if selected, sent to RIVM for MC-PCR testing.

Table 3: Number of slaughterhouses visited per country

Country	**Slaughterhouses**
United Kingdom	3 slaughterhouse for adult cattle, 1 slaughterhouse for calves
Italy	8 slaughterhouses from 4 regions (NW, NE, C, S), (3 regions: 1 slaughterhouse; 1 region: 5 slaughterhouses)
Romania	4 slaughterhouses and 9 samples from backyard slaughtering
The Netherlands	1 slaughterhouse for adult cattle, 1 slaughterhouse for calves

1.1.2.2. 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 according to appendix 6.1.3.

1.1.2.3. Mouse bioassay

Cattle were tested by mouse bioassay of the liver in the country of sample collection (one laboratory per country). Trypsin digestion of liver and inoculation in two mice per digest was performed as described in appendix 6.1.1. The development of antibodies against *T. gondii* in mice was determined by serology on day 28 in Italy and at post mortem (day 42) in the UK, Romania and the Netherlands. Each laboratory was free to choose a method for antibody detection in the mice. Samples from NL and UK were tested by ID.Vet ELISA (ID Screen® toxoplasmosis indirect multi-species) and, if

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necessary, sent to Reims for confirmation by MAT; all mouse sera from Romania were sent to Reims for MAT without any prior testing; sera from Italy were tested using the commercially available modified agglutination test (Toxoscreen DA, BioMerieux). DNA was isolated from mouse brain homogenates according to appendix 6.1.2.1 and tested by qPCR targeting a 529bp Repeated Element (RE) (appendix 6.1.2.3), or, in RO, by conventional PCR on the 529bp RE DNA fragment followed by gel electrophoresis (Homan et al., 2000). 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.

1.1.2.4. PCR on liver digest

DNA was isolated from liver digests using the Nucleospin kit (Machery-Nagel) as described in appendix 6.1.2.1. Samples were subsequently tested by 529bp RE qPCR or, in RO, by conventional PCR on the 529 bp DNA fragment (Homan et al., 2000). This was performed in the country of sample collection. PCR was considered positive using the criteria described for PCR on mouse brains.

1.1.2.5. 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 100 cattle (25/country) negative in bioassay and PCR on the digest, but irrespective of their serological result, were tested by MC-PCR. MC-PCR was performed as described in appendix 6.1.2.2 and 6.1.2.3. PCR was considered positive using the criteria described for PCR on mouse brains (section 1.1.2.3).

1.1.2.6. 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). Interpretation of kappa-values was based on the categories defined in Appendix B. For this comparison, mouse bioassay, PCR on liver digest, and MC-PCR on diaphragm were considered separately.

1.1.3. Results

1.1.3.1. Origin of the samples

The proposed sample sizes were reached in all four countries. The sex and age distribution of the cattle is presented by country in Table 4. As expected, slaughtered calves were mainly male, whereas slaughtered adult cattle were mainly female. Calves slaughtered in the UK were younger than those from the other countries. In Romania, most animals came from backyard farming.

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Table 4: Mean age and sex of cattle sampled in Italy (IT), the Netherlands (NL), Romania (RO) and the United Kingdom (UK)

			Calves		**Adults**	
			Count	Mean age (months)	Count	Mean age (months)
Country	IT[a]	NA	0	.	1	135
		Female	2	9	32	52
		Male	48	7	16	17
		Total	50		49*	
	NL	Female	7	6	50	66
		Male	43	7	0	.
		Total	50		50	
	RO	Female	15	4	44	106
		Male	35	5	6	23
		Total	50		50	
	UK	Female	0	.	37	31
		Male	17	1	48	25
		Total	17[#]		85	
Total		Female	24		163	
		Male	143		70	
		Total	167		233	

[a] One animal was not registered at bovine register office

*Age and sex were missing for one adult (based on slaughterhouse) from Italy, thus 50 calves and 50 adults have been sampled.

[#] Sixteen calves were sampled in designated slaughterhouse for calves, these were between 0 and 2 months old, one 12-month old male was sampled at a slaughterhouse for adult cattle.

1.1.3.2. Detection of antibodies against *T. gondii* in cattle

Antibodies were detected by MAT in 14.9% of cattle (Table 5). Titers were low, and the maximum titer was 1:200 for one 25 months old cow from the UK. The seroprevalence was higher in older cattle (Pearson's χ^2 *p*-value=0.011) (Table 6).

Table 5: Modified agglutination test (MAT) titers and classification (cut-off value ≥1:6) for cattle from the United Kingdom (UK), Italy (IT), Romania (RO) and the Netherlands (NL)

MAT	UK	IT	RO	NL	
negative	90	96	75	81	342
positive	12	4	25	19	60
1:6	1	1	14	6	22
1:10	3	1	1	6	11
1:25	4	2	6	1	13
1:50	3	0	2	5	10
1:100	0	0	2	1	3
1:200	1	0	0	0	1
	102	100	100	100	402

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Table 6: Modified agglutination test (MAT) titers and classification (cut-off value ≥1:6) for cattle by age

MAT	Adult	Calf	Total
negative	191	151	342
positive	44 (18.7%)	16 (9.6%)	60 (14.9%)
1:6	15	7	22
1:10	10	1	11
1:25	9	4	13
1:50	8	2	10
1:100	1	2	3
1:200	1	0	1
	235	167	402

1.1.3.3. Direct detection of *T. gondii* in cattle

The presence of *T. gondii* was demonstrated in the tissues of 13 out of 402 cattle (Table 7): 4 from IT, 7 from RO and 2 from NL. Positive results were always limited to one direct detection method per cattle, i.e. there was no overlap in positive results from mouse bioassay, PCR on liver digest and MC-PCR on diaphragm. A description of the positive animals is provided in Table 8. As the number of direct detection positives is low, statistical evaluation of direct detection positivity by age, sex or type was not considered useful.

Table 7: Detection of *T. gondii* in the tissues of cattle (positive/total) from the United Kingdom (UK), Italy (IT), Romania (RO), and the Netherlands (NL)

	UK	**IT**	**RO**	**NL**	**total**
Mouse bioassay	0/85	4/100	0/100	2/100	6/385
serology mice	0/84	0/100	0/95	0*/100	0/379
PCR mouse brain	0/85	4/100	0/100	2/100	6/385
PCR digest	0/100	0/100	0/100	0/100	0/400
MC-PCR#	0/34	0/30	7/44	0/43	7/151

*There were 2 mice that tested positive in ID.vet ELISA, but these were negative in MAT
MC-PCR was performed on diaphragm from 25 cattle regardless of MAT result and negative by mouse bioassay and PCR on the digest, and additionally on all cattle positive in mouse bioassay, PCR on digest or MAT.

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Table 8: Characteristics of cattle from the United Kingdom (UK), Italy (IT), Romania (RO), and the Netherlands (NL) found positive by a direct detection method for *T. gondii*

Animal ID	Country	Age (months)	Sex	Type	Direct detection	Indirect detection
B105	IT	67	F	Holstein-Friesian (dairy)	1 mouse PCR+	neg
B176	IT	15	F	Holstein-Friesian (dairy)	1 mouse PCR+	neg
B178	IT	18	M	Piemontese (beef)	2 mice PCR+	neg
B179	IT	18	M	Piemontese (beef)	1 mouse PCR+	neg
B246	RO	154	F	Cross-breed	MC-PCR+	1:6
B256	RO	8	M	Cross-breed	MC-PCR+	neg
B257	RO	2	M	Cross-breed	MC-PCR+	neg
B258	RO	12	M	Cross-breed	MC-PCR+	1:100
B259	RO	3	F	Dairy	MC-PCR+	neg
B260	RO	1	F	Dairy	MC-PCR+	neg
B263	RO	10	M	Cross-breed	MC-PCR+	1:6
B351	NL	107	F	NA	1 mouse PCR+	1:6
B367	NL	128	F	NA	1 mouse PCR+	1:50

Mouse bioassay: Mice were tested serologically and by PCR on the brain, and if either of these methods gave a positive result in one of the two mice inoculated with the same liver digest, the mouse bioassay is scored positive in Table 9. For sixteen calves from the UK 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. For 22 cattle with a negative mouse bioassay result, one or more results were missing (e.g. a serological result would be missing when a mouse was found dead). With the exception of one mouse bioassay from IT (B178) in which both mice tested positive by qPCR on the brain, all positive mouse bioassays are based on one positive result (PCR or serology for one mouse). Two mouse bioassays from the Netherlands were initially considered positive based on a positive result for one mouse each in the ID.Vet ELISA. However, these mice were serologically negative in MAT and confirmed negative in immunoblot when further tested for antibodies against p30 (Tg-SAG1) antigen at Friedrich-Loeffler-Institut, therefore these bioassays are considered negative.

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 from a selection of cattle. Twenty-five samples were tested irrespective of the results in other methods. The diaphragms from all cattle positive in mouse bioassay, digest PCR or MAT were additionally tested; with the exception of one Romanian cattle with a MAT titer of 1:6, which was omitted by mistake. Seven Romanian cattle gave a positive MC-PCR result. These cattle were negative in mouse bioassay and by PCR on the liver digest, and only two showed an antibody response (MAT 1:100 and 1:6).

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

In tables 9 to 11 the direct detection results are presented by serological status. A MAT titer ≥1:6 was considered positive. There is 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

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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. The direct detection rate of *T. gondii* in seropositive cattle is low (3.3% by mouse bioassay and 5.1% by MC-PCR) and similar to the detection rate in seronegative cattle (1.2% by mouse bioassay and 4.3% by MC-PCR) (Fisher's Exact test, p-value = 0.237 and p-value =1.000).

Table 9: Demonstration of viable *T. gondii* in liver by mouse bioassay (serology or PCR on brain) (positive/total) in cattle from UK, IT, RO and NL, stratified by serological status in MAT (cut-off ≥1:6)

	Mouse bioassay				
MAT	UK	IT	RO	NL	total
negative	0/73	4/96	0/75	0/81	4/325 (1.2%)
positive	0/12	0/4	0/25	2/19	2/60 (3.3%)
	0/85	4/100	0/100	2/100	6/385 (1.6%)

Table 10: PCR-based detection of *T. gondii* DNA in trypsin-digest of liver (positive/total) in cattle from UK, IT, RO and NL, stratified by serological status in MAT (cut-off ≥1:6)

	PCR digest				
MAT	UK	IT	RO	NL	total
negative	0/89	0/96	0/75	0/81	0/341 (0.0%)
positive	0/12	0/4	0/25	0/19	0/60 (0.0%)
	0/101	0/100	0/100	0/100	0/401 (0.0%)

Table 11: Detection of *T. gondii* DNA in 100g of diaphragm by magnetic capture and qPCR (positive/total) in cattle from UK, IT, RO and NL, stratified by serological status in MAT (cut-off ≥1:6)

	MC-PCR				
MAT	UK	IT	RO	NL	total
negative	0/22	0/26	4/20	0/24	4/92 (4.3%)
positive	0/12	0/4	3/24*	0/19	3/59 (5.1%)
	0/34	0/30	7/44	0/43	7/151 (4.6%)

*One seropositive cattle from Romania was not tested by MC-PCR

1.1.4. Discussion and conclusion

Serum, liver and diaphragm samples have been collected from cattle slaughtered in four countries. In all countries the targeted sample size of 100 cattle per country has been reached. In NL, RO and IT, adult cattle and calves were sampled in equal proportions. It was realised beforehand that this would not be feasible in the UK, therefore we aimed to sample 16 calves and 84 adult cattle. Unfortunately, all mice inoculated with calf liver in the UK died soon after inoculation. Therefore mouse bioassay results for UK calves are lacking, with the exception of one cattle nearly 12 months old that was sampled by chance in a slaughterhouse for adult cattle.

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Cattle sera were tested by MAT. All livers were digested using trypsin, tested by PCR and inoculated into mice. To detect infection in mice, the mice were examined serologically and mouse brains were tested by PCR. A selection of diaphragms from negative cattle and all diaphragms of bioassay or serologically positive cattle were tested by magnetic capture and qPCR. It was our aim to harmonize the PCR methods in all involved countries, therefore the qPCR for the 529 bp repeat element (RE) already available in NL and UK was set up in RO and IT. In RO, PCR contamination issues arose, and this could not be solved in time. As it was unclear at what point in the process contamination occurred and contamination during sample preparation could not be ruled out, sending the samples to another institute was not a reliable solution. As an alternative solution, conventional PCRs were performed in RO without any further issues. The sensitivity of this method was tested and found to be similar to the qPCR for the 529 bp RE performed in the other countries, therefore, the difference in methods should not hamper the validity of the comparison of the PCR results from the different countries.

Overall, 13 animals were considered direct detection-positive, and for 6 out of 385 cattle this conclusion is based on mouse bioassay (1.6%), indicating the presence of viable tissue cysts and potential risk for consumers. These six positive mouse bioassays concerned two mice from NL (corresponding to two cattle) and 5 mice from IT (corresponding to 4 cattle), which were positive in PCR on the brain, but negative in MAT. No clinical signs of toxoplasmosis were observed in the mice, only one liver digest resulted in infection in both mice, and the PCRs carried out directly on the digest were always negative. Overall, these mouse bioassay results strongly suggest that even when present, the parasite concentration is generally low in bovine liver. As a consequence, low inoculation doses have resulted in low grade infections in mice, low parasite loads in mouse brain homogenates and failure to elicit a detectable antibody response in mice.

All seven cattle positive in MC-PCR on the diaphragm originated from Romania. The positive PCR reactions were confirmed by gel electrophoresis, as Cq-values were high (between 36 and 40 cycles) and not always repeatable. This indicates a low concentration of *T. gondii* DNA in diaphragm. For these samples the presence of non-viable parasites or DNA cannot be ruled out, therefore these results do not provide an indication of risk for consumers.

The results for all 13 direct detection positive cattle are based on PCR, either on mouse brain homogenates or after magnetic capture-based DNA isolation from diaphragms. To exclude potential contamination, appropriate blank controls were included during DNA isolations and PCR runs, and always confirmed negative. Non-specific amplification was excluded by considering only samples with an amplification curve similar to the positive controls and a Cq-value below 40. For samples scoring positive between cycle 35 and 40, the amplicon size was checked by gel electrophoresis. Due to the low DNA concentrations in the amplified samples, it was not possible to get further confirmation of the presence of *T. gondii* DNA based on single copy genes suitable for PCR-based genotyping.

As expected, the number of animals positive by a direct detection method was limited. Nevertheless, the number of positives was sufficient to demonstrate the lack of concordance between the presence of antibodies against *T. gondii* and direct detection of the parasite using either mouse bioassay, qPCR on digest or MC-PCR. In addition, the probability to directly detect *T. gondii* in seropositives versus seronegatives was found to be similar, demonstrating that serological testing does not provide information about the presence of *T. gondii* in cattle. The concordance between direct and indirect detection can be influenced by the choice of methods. In this case, the MAT was selected for the detection of antibodies in cattle. This is a species-independent serological test that is commonly used with bovine sera. The test has been shown a proper serological tool in the follow-up of experimentally infected cattle (Dubey et al., 1985), but has not been evaluated in depth for use with cattle sera in a

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field situation. Therefore, the lack of concordance might be caused by misclassification based on MAT results. However, no serological assay with proven sufficient performance is currently available for cattle. Cattle sera have additionally been tested for antibodies against p30 (Tg-SAG1) by immunoblot at Friedrich-Loeffler-Institut (data not shown), providing the possibility to discriminate more easily between specific and non-specific reactions. These results do not correlate well with MAT or overall direct detection. Romanian cattle sera have also been tested using IDEXX Toxotest Ab Test (TXT1135T) (data not shown), again with low concordance with MAT and overall direct detection.

For direct detection, three different methods were chosen. The results of these three methods do not overlap, demonstrating that, also in this case, the choice of method influences the concordance between direct and indirect detection. The observed discordance may have several possible explanations: Diaphragm samples were tested by MC-PCR, whereas liver digests were tested by PCR and inoculation in mice, and different tissues are not necessarily equally infected with *T. gondii*. Liver digests were tested by PCR and mouse inoculation, but no positives were identified by PCR. This is likely due to a lower sensitivity of the PCR compared to mouse bioassay, as only a 200 µl sample is processed for DNA isolation and a fraction of that tested in PCR, whereas 1 ml of tissue digest is inoculated in two mice per sample. This lower sensitivity of PCR on digests has also been observed in the calf infection experiment (Table 14, section 1.2.3.3). The combination of direct detection methods used has been optimized to match budget availability. Use of a more sensitive direct detection method could increase the concordance between direct and indirect detection, if the additional positives mainly came from the seropositive category. However, in the current study the lack of concordance is consistent whichever direct detection method is considered, therefore an increase in concordance is not expected.

The liver was chosen as predilection site and diaphragm as representative of edible tissue, which resulted in 6 cattle tested positive by mouse bioassay on liver and 7 other cattle positive by MC-PCR on diaphragm. From the results presented here, it appears that cattle are unlikely to be infected to a detectable level in both tissues simultaneously. However, because different methods were used for the two tissues and not all cattle were tested by MC-PCR, it is not possible to conclude that one tissue is more likely to be infected than the other or to conclude that one method is performing better than the other.

In summary:

1. *T. gondii* has been detected in 13 out of 402 cattle and in 6 of these detection was based on mouse bioassay (1.6%), indicating the presence of viable parasites.

2. The discordance between results of the different direct detection methods, the lack of clinical toxoplasmosis in mice or corresponding serological and PCR positivity for mice, and the low DNA concentrations detected by qPCR in mouse brains and bovine diaphragms, demonstrate that the number of parasites in bovine liver and diaphragm is low.

3. The data are not suitable to compare the parasite load in liver and diaphragm or the performance of mouse bioassay and MC-PCR.

4. The lack of concordance between MAT results and the different direct detection methods employed, indicates that there is no useful relationship between the detection of antibodies and presence of *T. gondii* in cattle. Therefore, MAT appears unsuitable to obtain an estimate of the prevalence of viable *T. gondii* in cattle and does not provide an indication of the risk for consumers.

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5. Misclassification due to the particular direct or indirect detection methods used in the study can not be ruled out and may have influenced the lack of concordance. However, the discordance is present whichever direct detection method is considered, and could not be resolved by the use of other serological assays. For that reason, the lack of concordance between methods likely represents a true lack of correlation between the presence of antibodies and the presence of (viable) *T. gondii* in cattle.

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1.2. Experimental infection with *Toxoplasma gondii* in calves

1.2.1. Introduction

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. Therefore, six calves seronegative for *T. gondii* were orally inoculated with oocysts of the M4-strain of *T. gondii* (a type II strain isolated from a case of ovine toxoplasmosis in Scotland). Post-mortem samples were collected 6 weeks post inoculation from heart, brain, liver, diaphragm, masseter muscle, tongue and various other edible tissues. The presence of infective parasites was determined by mouse bioassay and parasite concentration was estimated by MC-PCR. Previous research has shown an oocyst challenge of around 10^4 to 10^5 oocysts is not likely to result in detection of *T. gondii* from bovine tissues by mouse bioassay (Dubey and Thulliez, 1993; Esteban-Redondo et al., 1999). In this experiment a challenge dose of 10^6 *T. gondii* oocysts was used, as detection of the parasite in bovine tissues has been shown following challenge with 0.75 x10^6 oocysts (Munday, 1978).

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 following hypotheses were tested:

1. Tissue cyst formation is detected within tissues intended for human consumption, a potential risk to humans.

2. Predilection sites for the parasite in cattle can be identified

1.2.2. Material and Methods

1.2.2.1. Cattle

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 one week prior to inoculation. All calves were showing signs of respiratory problems prior to inoculation. Calves were housed together and after weaning were fed a commercial calf feed with water available *ad libitum*.

1.2.2.2. 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 euthanised 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

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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.

1.2.2.3. 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). Trypsin digestion and mouse inoculation was carried out as described in appendix 6.1.

1.2.2.4. DNA extraction from mouse brain and tissue digest

DNA extraction from individual mouse brains and tissue digests were carried out as described in appendix 6.1

1.2.2.5. 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) as described in appendix 6.1.3.

1.2.2.6. 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.

1.2.2.7. MC-PCR

100g tissue pools were prepared as described for mouse bioassay and sequence-specific DNA isolation using magnetic capture was performed as described in appendix 6.1.2.2. Remaining tissue samples of liver, heart, diaphragm, triceps femoralis, semitendinosus and masseter were tested individually.

1.2.2.8. *T. gondii* 529bp RE quantitative PCR

DNA extracted from mouse brains, tissue digests and DNA samples isolated by magnetic capture were tested by qPCR as described in appendix 6.1.2.3.

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1.2.3. Results

1.2.3.1. Temperature and clinical signs

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°C, calf 115) (Fig. 1). The rectal temperature of calf 109 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.

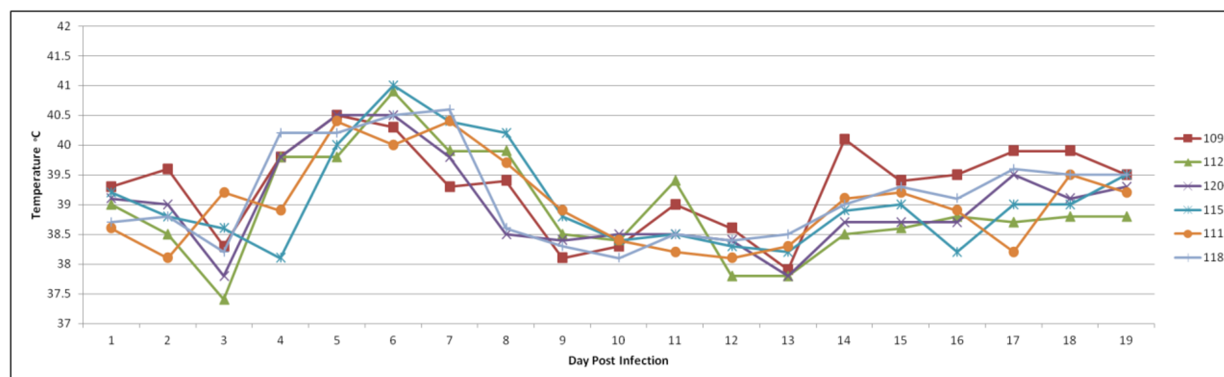

Figure 2: Rectal temperatures of all calves between day 1 – 19 of the experiment

1.2.3.2. *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 (Table 12).

Table 12: *T. gondii* serology of experimental calves by MAT

Day p.i.	Calf Number					
	109	112	120	115	111	118
0	0	0	0	0	0	0
7	0	0	0	0	0	0
14	50	10	6	100	3	25
21	1600	1600	400	800	800	1600
28	1600	1600	1600	1600	800	1600
35	1600	800	1600	1600	800	1600
42	X	800	800	800	1600	1600

X = Animal died, no sample available for testing.

1.2.3.3. 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

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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 (50-1, 50-3, 51-1, 51-3, 49-1 and 49-3) from pool two were culled early (d12) 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 13). 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 (from mice; 49-10, 49-30, 50-3, 52-1, 53-1, 53-3, 53-10, and 53-30), were further tested using the p30 (Tg-SAG1) immunoblot at Friedrich-Loeffler-Institut, and an additional three samples (52-1, 53-3, 53-30) were identified as positive.

Table 13: Detection of *T. gondii* from pooled calf tissues by mouse bioassay, qPCR on tissue digests and MC-PCR

	Mouse 1				Mouse 2				Inocula	Calf Tissues	
Tissue	Mouse No.	Cull Date (dpi)	ELISA Result (%SP)	qPCR (cq)	Mouse No.	Cull Date (dpi)	ELISA Result (%SP)	qPCR (cq)	qPCR (cq)	MC-qPCR (cq)	Calf Pool
liver	44-1	42	neg	neg	44-3	42	neg	neg	neg	neg	Pool 1 = x2 animals 112 & 120
masseter	44-10	42	neg	neg	44-30	42	neg	neg	neg	neg	
tongue	45-1	8	No sample	neg	45-3	42	neg	neg	neg	neg	
heart	45-10	42	neg	neg	45-30	42	neg	neg	neg	neg	
brain	46-1	6	No sample	neg	46-3	3	neg	neg	neg	neg	
diaphragm	46-10	42	neg	neg	46-30	42	neg	neg	neg	neg	
Left *Tricep femoralis* (forelimb)	47-1	42	neg	neg	47-3	42	neg	neg	#37.20	neg	
Left *semitendinosus* (hindlimb)	47-10	42	neg	neg	47-30	42	neg	neg	neg	neg	
Psoas major (fillet)	48-1	42	neg	neg	48-3	42	neg	neg	neg	neg	
Longissimus dorsi (sirlion)	48-10	42	neg	neg	48-30	42	neg	neg	neg	neg	
liver	53-1	42	neg	33.79	53-3$	42	neg	33.83	neg	34.40	Pool 2 = x3 animals 115, 111 & 118
masseter	50-10	42	neg	neg	50-30	42	neg	neg	neg	35.54	
tongue	50-1	12	24	19.66	50-3	12	neg	19.04	33.37	32.34	
heart	49-10	42	neg	34.01	49-30	42	neg	33.53	neg	32.84	
brain	51-1	12	12	21.56	51-3	12	13	21.70	neg	neg	
diaphragm	49-1	12	8	23.09	49-3	12	16	21.47	29.04	34.16	
Left *Tricep femoralis* (forelimb)	52-1$	42	neg	33.60	52-3	42	neg	neg	neg	34.63	
Left *semitendinosus* (hindlimb)	52-10	42	285*	18.43	52-30	42	230*	19.87	33.92	32.54	
Psoas major (fillet)	53-10	42	neg	18.13	53-30$	42	neg	19.90	neg	34.09	
Longissimus dorsi (sirlion)	51-10	42	neg	neg	51-30	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.
$Positive by p30 (Tg-SAG1) immunoblot.

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

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118 (Table 14). Not enough material of other tissues remained for several calves, therefore these tissues have not been tested individually.

Table 14: Detection of *T. gondii* in calf tissues by MC-PCR (volume of tissue tested and Cq-value)

Calf	Pool	Heart		Liver		Diaphragm		Triceps femoralis		Semi-tendinosus		Masseter	
109	Excl	82g	neg	85g	neg	74g	36.91	91g	neg	100g	neg	81g	neg
112	1	91g	37.91	100g	neg	8g	neg	70g	neg	100g	neg	95g	neg
120	1	77g	neg	95g	neg	48g*	neg	55g	neg	57g	neg	69g	neg
115	2	100g	neg	100g	neg	56g	neg	94g	neg	94g	neg	50g	neg
111	2	100g	neg	100g	neg	66g	neg	100g	neg	100g	neg	37g	neg
118	2	89g	neg	96g	neg	NA	NA	65g	32.27	98g	32.63	69g	29.45

* For calf 120 the diaphragm was tested in two separate portions, first 13g and later an additional 35g. Both portions tested negative by MC-PCR.

1.2.4. Discussion and conclusion

Small intestine and liver were identified as predilection sites for *T. gondii* in cattle by extensive review of the literature. Skirt steak, lymph nodes, thigh muscle and top round steak also did very well in a limited number of studies (see systematic review report and also Table 1 in Section 1.1.1). In the current study additional information is obtained by performing mouse bioassay, qPCR on digest and MC-PCR on the most relevant types of tissues of experimentally infected calves.

Serological detection of *T. gondii* in calves was performed using MAT, with all calves demonstrating high titers from 21 days post inoculation onwards (Table 12), demonstrating that inoculation has resulted in successful infection in all six calves. These results are similar to what was observed in experimental cattle by Munday (1978) where both IFAT and dye test were used to monitor *T. gondii* titres. Despite all calves showing seropositivity, tissue cysts could not be detected as readily from all animals. Most of the direct detection was performed on pooled tissues. Unlike pool 2 with three calves, pool 1 tissues were comprised from only two calves as during the experiment one calf died due to pneumonia. All calves had been showing signs of pneumonia on arrival at Moredun Research Institute and were treated accordingly prior to inoculation, however this calf never seemed to fully recover. Therefore, as pool 1 had only tissues from two calves this is likely to have affected the results, particularly if these animals did not have a high parasite burden.

qPCR on inocula did not appear to be as sensitive as MC-PCR on the calf tissues directly or mouse bioassay of tissues, and was only positive for pool 2 tongue, diaphragm and semitendinosus digests. Surprisingly, parasite DNA was also detected in the triceps inocula of pool 1 calves, whereas none of these pooled tissues tested positive in mouse bioassay or MC-PCR.

Mouse bioassay was performed by inoculation of 1ml of the same tissue digest used for qPCR into two mice per digest. As the volume inoculated into the mice was larger than the volume used for DNA isolation it is not surpising that more positives were detected by mouse bioassay. Mouse bioassay results are based on clinical signs, detection of antibodies in the mice and qPCR on mouse brains. These results did not always overlap. On days 6 and 8, two mice (46-1 and 45-1) from pool 1 that were used in the bioassay had to be euthanised due to signs similar to *T. gondii* infection, however, it was too early for detection of an IgG response and parasite DNA could not be detected from the brains of either of these mice. Mouse 45-1 had received tongue tissue inocula and mouse 46-1 brain tissue inocula, but qPCR of the inocula and MC-PCR of the tissue pool did not identify parasite DNA. It is

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not possible to make a final conclusion regarding the infection status of these mice and the corresponding calf tissues: These clinical signs may have been unrelated to *T. gondii* infection or the mice may still have been in the acute phase of *T. gondii* infection with tachyzoite replication but in which tissue cysts have not yet developed in the brain. Serological detection of *T. gondii* from mice used in the bioassay (using the ID.Vet ELISA) did not provide an accurate indication as to whether the mice were infected with the parasite (Table 13). Weak ELISA results for mice inoculated with tongue, brain and diaphragm digests can be explained by the need to cull early, leaving less time to develop a strong immune response. Mouse sera from eight mice from which the brains had been qPCR positive, but ELISA negative (49-10, 49-30, 50-3, 52-1, 53-1, 53-3, 53-10, and 53-30), were sent to Friedrich-Loeffler-Institut for re-testing using the p30 (TgSAG1) immunoblot. The TgSAG1 immunoblot identified a further three seropositive mice (52-1, 53-3 and 53-30), resulting in a better agreement between the different methods as the brains from these mice were also qPCR positive and the corresponding calf tissue they had been inoculated with was also positive by MC-PCR. Mouse bioassay is not a quantitative method and can only give an indication of the parasite load in the tested tissues. Considering only pool 2: mice inoculated with tongue, brain and diaphragm had to be culled early and the Cq-values were low; mice inoculated with semitendinosus muscle gave low Cq-values and had strong antibody responses; mice inoculated with psoas major muscle showed low Cq-values; mice inoculated with liver, heart and triceps muscle were positive with high Cq-values. Mice inoculated with masseter muscle and longissimus dorsi muscle remained negative.

Detection of parasite DNA by MC-PCR in pooled tissues agrees nicely with the mouse bioassay results. When examining the quantitative MC-PCR results from pools of specific tissues, tongue, semitendinosus muscle and heart had slightly lower Cq values (ranging from 32.34 to 32.84) than the other qPCR positive tissues (psoas major, diaphragm, liver, triceps and masseter with Cq values ranging from 34.09 to 35.54) (Table 13). In a 100% efficient realtime PCR amplified DNA will double with every cycle and 10-fold differences in initial DNA concentration will be 3.3 Cq-values apart. Even the difference in Cq-value between tongue and masseter indicates less than a 10-fold difference in DNA concentration and therefore our results do not suggest the presence of true predilection sites.

Based on pooled tissues, no conclusion can be made about the infection status of the individual calves, therefore remaining heart, liver, diaphragm, triceps femoralis, semitendinosus and masseter were tested individually by MC-PCR. Some discrepancy can be expected as a different part of the tissue is cut and used for the two magnetic captures, and the concentration of tissue cysts is expected to be low in cattle. The results show that parasites have also disseminated to tissues in calf 109 (diaphragm) and 112 (heart). However, only one (118) of the pool 2 calves tested positive, but not in heart or liver (no diaphragm of calf 118 remained). Although it cannot be ruled out that any of the other pool 2 calves harbored *T. gondii* tissue cysts, these result suggest that the positive results for pool 2 are likely attributed to calf 118 alone.

In summary the results show that:

1. After oral inoculation with 10^6 oocysts of calves, both viable *T. gondii* and DNA were detected in various tissues including meat cuts.

2. Semitendinosus and tongue gave consistent mouse bioassay results and were among the samples with lower Cq-values in MC-PCR, but many other tissues of pool 2 also tested positive in both methods, therefore no clear predilection sites were identified.

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1.3. *Toxoplasma gondii* vaccination (S48) and challenge experiment in sheep

1.3.1. Introduction

In the UK, vaccination of ewes using Toxovax is applied to prevent *T. gondii* related abortions. These animals will be seropositive, but are likely to be (partially) protected against tissue cyst formation. This is important as in these ewes the correlation between serology and detection of tissue cysts is likely to be different, and vaccination is a potential control measure. Experimental infections of Toxovax-vaccinated sheep have been carried out at Moredun Research Institute, and results from nested PCR (based on the ITS1 sequence of the 18S rDNA) (Katzer et al., 2014), indicate a reduction, but not a complete prevention of tissue cyst formation in these sheep. Using tissues already collected and stored from this experiment, tissue samples were tested by MC-PCR to quantify the anatomical distribution of tissue cysts in these sheep and compare it to tissue cyst formation in the unvaccinated controls.

Aim of the study: using tissues which have been collected and stored from the above experiment the following hypotheses were tested:

1. Tissue cyst formation by the oocyst stage of the parasite is detected within tissues intended for human consumption, a potential risk to humans.

2. Vaccination reduces the number of tissue cysts or prevents tissue cyst formation, and therefore help producing a safer lamb.

1.3.2. Material and Methods

1.3.2.1. Study design

Animals (33 Suffolk lambs, 4 weeks of age) were infected orally with $5x10^5$ oocysts of M4 *T. gondii*, a type II strain which is held at the Moredun Research Institute, $1.2x10^5$ S48 tachyzoites for vaccination, or a control inoculum of media. The high challenge dose was used to ensure that we would be able to identify which tissues the parasite disseminated to (predilection sites) and based on previous studies in lambs at Moredun Research Institute (Benavides et al., 2011) a challenge dose of $5x10^5$ oocysts was sufficient to allow the parasite to establish within the host without causing unnecessary suffering (the dose administered to different species is of particular importance as parasite virulence can differ across different host species). There were four experimental groups (as shown in the Table 15). One month before the challenge, Group 2 animals were vaccinated with the incomplete *T. gondii* S48 strain, the same strain which is used in the commercial vaccine Toxovax. During the experiment, the animals were observed daily. Prior and after the infection, rectal temperatures were taken daily until day 14 post infection. Blood samples were collected twice weekly from 1 day before challenge until the end of the experiment by jugular venepuncture. Lambs were culled at day 14, 28 and 42 post-infection. For this project, only the animals from group 1 and 2 that were culled at 28 and 42 days post infection were tested.

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Table 15: Grouping of lambs in *Toxoplasma gondii* vaccination and challenge experiment

Group	n	Treatment	Frequency of treatments	Purpose
1	12	Infection with $5x10^5$ M4 *T. gondii* oocysts. Animals culled at 14, 28 and 42 days post infection (4 animals at each timepoint)	1	To establish infection with *T. gondii.* This group will allow to study the immune response and cyst formation by the parasite
2	12	Vaccination with $1.2x10^5$ S48 tachyzoites 4 weeks before infection then infection with $5x10^5$ M4 *T. gondii* oocysts. Animals culled at 14, 28 and 42 days post infection (4 animals at each timepoint)	1	To evaluate the effect of the immunization over the cysts formation and immune response.
3	6	Vaccination without challenge ($1.2x10^5$ S48 tachyzoites). Animals culled at 14, 28 and 42 days post infection (2 animals at each timepoint)	1	Control group. Vaccination should not cause any pathology or tissue cysts.
4	3	Control animals inoculated with the dilution media. Animals culled at 14, 28 and 42 days post infection (1 animal at each timepoint)	1	Control group with no infection and no vaccination.

1.3.2.2. MC-PCR

MC-PCR is described in appendix 6.1. As the lamb experiment was completed in 2010 at Moredun Research Institute, not all samples were available for testing, with only 5 g of each tissue still stored at -20°C. Therefore, either 10 g pools of tissue from two animals or 5 g tissue from individual animals was processed. Lysis buffer volumes were adjusted accordingly (2.5 ml/gram of tissue). Further processing was completed on either 12 ml (for 10 g samples) or 10 ml (for 5 g samples) as described for the MC-PCR protocol in appendix 6.1.

1.3.3. Results

Only MC-PCR was performed within this project. Full details of the results with other methods have been published outside this EFSA project (Katzer et al., 2014).

1.3.3.1. Serology

By day 21 all lambs from groups 2 and 3 were seropositive for *T. gondii* by ELISA.

1.3.3.2. Molecular detection of *T. gondii* from lamb tissues

Comparison between previous molecular detection of the parasite (ITS1 PCR) and the detection carried out as part of the EFSA project (MC-PCR), shows that all tissues from group 1 (oocyst challenged animals), which were positive by ITS1 PCR are also positive by MC-PCR (see Table 16). In addition, one tissue (loin) from a lamb in group 1, which was ITS1 negative tested positive by MC-PCR.

All tissues (chop, loin, forelimb and hindlimb) from group 2 lambs (vaccinated and challenged) were negative by both ITS1 PCR and MC-PCR (Table 16). Further details of all ITS1 PCR results are available as published data (Katzer et al., 2014).

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Table 16: Molecular detection of *T. gondii* from lamb tissues by ITS1 PCR and MC-PCR

<table>
<tr><th rowspan="2">Treatment</th><th rowspan="2">Group</th><th rowspan="2">Animal</th><th colspan="6">Tissue: MC-qPCR cq value</th><th rowspan="2">Day PM</th><th rowspan="2"></th></tr>
<tr><th>Longissimus dorsi (chop)</th><th>Psoas major (loin)</th><th>Right Tricep femoralis (forelimb)</th><th>Left Tricep femoralis (forelimb)</th><th>Right Semitendinosus (hindlimb)</th><th>Left Semitendinosus (hindlimb)</th></tr>
<tr><td rowspan="4">Ooocyst Challenged</td><td rowspan="4">1</td><td>6626</td><td>25.25 (+)</td><td>n/a</td><td rowspan="2">22.64 (++)</td><td rowspan="2">22.95 (++)</td><td rowspan="2">24.33 (++)</td><td>n/a</td><td rowspan="2">28</td><td rowspan="2">pool 2 animals per tissue - day 28 G1</td></tr>
<tr><td>6749</td><td>n/a</td><td>25.72 (+)</td><td>n/a</td></tr>
<tr><td>6641</td><td rowspan="2">24.43 (++)</td><td>*26.98 (-)</td><td rowspan="2">23.99 (++)</td><td rowspan="2">#28.23 (-+)</td><td rowspan="2">24.06 (++)</td><td>n/a</td><td rowspan="2">42</td><td rowspan="2">pool 2 animals per tissue - day 42 G1</td></tr>
<tr><td>7358</td><td>n/a</td><td>24 (+)</td></tr>
<tr><td rowspan="4">Vaccinated and oocyst challenged</td><td rowspan="4">2</td><td>6603</td><td>n/a</td><td>0 (-)</td><td>n/a</td><td>n/a</td><td>0 (-)</td><td>n/a</td><td rowspan="2">28</td><td rowspan="2">pool 2 animals per tissue - day 28 G2</td></tr>
<tr><td>6703</td><td>n/a</td><td>n/a</td><td>n/a</td><td>n/a</td><td>n/a</td><td>n/a</td></tr>
<tr><td>6941</td><td rowspan="2">0 (--)</td><td rowspan="2">0 (--)</td><td>0 (-)</td><td rowspan="2">0 (--)</td><td rowspan="2">0 (--)</td><td rowspan="2">0 (--)</td><td rowspan="2">42</td><td rowspan="2">pool 2 animals per tissue - day 42 G2</td></tr>
<tr><td>6981</td><td>n/a</td></tr>
</table>

Only 5g of tissue available for MC-PCR (pools = 10g total, individual tissues = 5g)
Details of duplicate ITS1 PCR results in brackets (+ = positive, - = negative). All individual samples in group 1 were ITS1 positive except: *6641 (loin) ITS1 negative but MC-PCR positive, #6641 ITS1 negative but pooled sample (forelimb) is MC-PCR positive, n/a: no sample available for MC-qPCR. All individual tissues in group 2 were ITS1 negative.

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1.3.4. Discussion and conclusion

As can be seen from the results in Table 16, parasite DNA could not be detected from vaccinated and challenged animals (group 2) by both the conventional ITS1 PCR and MC-PCR on specific tissues (chop, loin, forelimb and hindlimb). This result demonstrates that vaccination of lambs with the S48 strain of *T. gondii* can reduce the parasite burden and formation of tissue cysts after challenge infection in lambs. The ITS1 PCR results of this experiment are described in detail in the published manuscript (Katzer et al., 2014).

When comparing the MC-PCR results with the ITS1 PCR, the MC-PCR appeared to be more sensitive, detecting a sample which was previously ITS1 negative in group 1 (day 42). This result may be due to the larger sample used for MC-PCR (5g), compared to 1g of tissue for ITS1 PCR. In addition, it can also occur by chance as different parts of the tissue samples were used for sample preparation. It is also interesting to note that the samples, that were ITS1 negative (6641 – loin and 6641/7358 – forelimb), were also the samples with the highest Cq values (26.98 and 28.23 respectively), therefore likely to have a lower concentration of parasite DNA. Specific tissues previously tested by ITS1 PCR (chop, loin, forelimb and hindlimb) from group 2 remained negative by MC-PCR.

In terms of the anatomical distribution of the parasite from the oocyst challenged animals (group 1), *T.gondii* DNA was detected by MC-PCR from all of the tissues tested within this EFSA project (chop, loin, forelimb, hindlimb) and the Cq values do not vary much between different tissues (22.64 – 28.23). This indicates a maximum difference in intial DNA concentration of approximately 50-fold. However, this maximum difference was observed between samples of the same tissue (triceps femoralis) from different pools of animals; therefore this difference should still be considered a normal range of variation of parasite load and does not indicate the presence of predilection sites among the different skeletal muscles tested.

In addition, the results also show the sensitivity of the MC-PCR technique as only a small amount of tissue was available (5 g) and the MC-PCR scaled down appropriately, however, the results correlated with what was previously observed with the ITS1 PCR. Also, as lambs were challenged $5x10^5$oocysts (therefore tissues likely to have a high parasite burden), it is reassuring to observe that the MC-PCR Cq values are also low, despite the small amount of tissue available for testing.

In conclusion, these results show that vaccination of lambs may reduce the transmission of *T. gondii* to humans via consumption of lamb and mutton. Within the vaccinated and challenged animals (group 2), parasite DNA was not detected from the tissues tested within the work completed specifically for this EFSA study (using MC-PCR), however, only small fractions of the lambs' total tissue volumes were tested and analysis of a broader range of tissues did detect *T. gondii* DNA by ITS1 PCR from distal jejunal/prescapular lymph nodes, liver, kidney and skeletal muscle from lambs euthanised on 28 and 42 days p.i. (Katzer et al., 2014). However, as mouse bioassay was not completed for this study, it is not possible to comment on the viability of tissue cysts, or whether the parasite DNA detected originated from tachyzoites of the S48 strain, as these were shown to persist in the tissues of vaccinated lambs at d42 (Katzer et al., 2014).

In summary:

1. *T. gondii* DNA was present at similar concentrations in various edible tissues of oocyst and tissue cyst inoculated lambs.
2. Vaccination using S48-strain tachyzoites reduces parasite load in edible tissues of lambs.

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1.4. Risk factors related to Toxoplasma gondii seroprevalence in indoor-housed Dutch dairy goats

Note: this section is presented as submitted to Preventive Veterinary Medicine for publication

1.4.1. Introduction

Toxoplasma gondii (*T. gondii*) is a zoonotic protozoan parasite that may cause serious disease in humans, especially when primary infection is acquired during pregnancy. In the Netherlands, *T. gondii* had the highest disease burden out of fourteen food-borne pathogens at both population and individual level (Havelaar et al., 2012).

In goats, toxoplasmosis causes abortion and stillbirth, and is thus a source of economic loss to goat farmers (de Moraes et al., 2011; van Engelen et al., 2014). Moreover, goats are considered important sources of human infection, especially for ethnic groups that commonly consume goat products (Tenter et al., 2000). Tissue cysts of *T. gondii* are responsible for infections via meat (Jones et al., 2012), and *T. gondii* tachyzoites can be excreted in goat's milk and can survive the raw fresh cheese-making process (Dubey et al., 2014). Infections due to the consumption of raw goat's milk have been reported (Sacks et al., 1982). There is a positive relationship between detection of antibodies against *T. gondii* in goats and presence of tissue cysts in their meat (Dubey et al., 2011). This correlation was also found between seropositivity and presence of *T. gondii* DNA in goat milk (Spišák et al., 2010). Therefore, the prevalence of antibodies in goats can be used to identify risk factors for infection but also gives an indication of the risk of infection for consumers through consuming raw goat products.

The reported percentage of *T. gondii* seropositive goats varied greatly among countries. In Europe, the seroprevalence of *T. gondii* in goats was estimated at 17% in Norway (Stormoen et al., 2012), 18.5% in north Portugal (Lopes et al., 2013), 30.7% in Greece (Tzanidakis et al., 2012), 25.1% in southern Spain (Garcia-Bocanegra et al., 2013), 52.8% in Romania (Iovu et al., 2012), and 60.6% in Italy (Mancianti et al., 2013). Regional variation of seroprevalence may be caused by differences in study population, study year and climate as well as differences in serological tests and criteria of cut-off value used in the test (Tenter et al., 2000). Reported risk factors associated with *T. gondii* infection in goats are age, presence of cats, management systems (i.e. extensive/intensive), source of drinking water and abortion history (Cavalcante et al., 2008; Gebremedhin et al., 2013; Tzanidakis et al., 2012; van Engelen et al., 2014).

In the Netherlands, several serological studies concerning *T. gondii* have been conducted, mainly in swine, poultry, cattle and sheep. The results showed a high seroprevalence in sheep (Opsteegh et al., 2010) compared to poultry and swine (van der Giessen et al., 2007; van Knapen et al., 1982). However, little is known concerning the seroprevalence of *T. gondii* in dairy goats. In 1998, epidemiological data on *T. gondii* infection in goats in the Netherlands were collected from ten Dutch goat farms, including three farms with a *T. gondii* abortion history. The mean seroprevalence was 47% (ranging from 5% to 90%) (Antonis et al., 1998). Since then, the number of dairy goats has nearly tripled, whereas the number of farms has decreased by 40% (CBS, 2015). This intensification of farming is associated with changes in farm management such as year round indoor-housing of dairy goats. In pigs and poultry, a lower seroprevalence of *T. gondii* infection was found in indoor farming systems than in outdoor farming systems (Maksimov et al., 2011; van der Giessen et al., 2007). Therefore, indoor-housed dairy goats are expected to have a low seroprevalence of *T. gondii*.

The objectives of this study were to determine the seroprevalence and to identify risk factors associated with *T. gondii* seroprevalence in Dutch indoor-housed dairy goats. A commercial indirect ELISA was used to test 1664 goat sera and serological results were analyzed using binary mixture models. Potential risk factors were evaluated by comparing Poisson, negative binomial and zero-inflated regression models.

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1.4.2. Materials and methods

1.4.2.1. Study population

In 2013, 451,377 goats were present in the Netherlands according to the Identification and Registration (I&R) database. Those animals were kept at 10,783 small goat farms (≤ 31 goats) and 546 professional goat farms (≥ 32 goats), of which 349 large dairy goat farms (≥ 50 goats). Most of the dairy goat farms use a deep litter housing system with dry straw as bedding material (Schimmer et al., 2011). This study focused on indoor-housed Dutch dairy goats at commercial farms with more than 100 goats that participated in an accreditation program for caprine arthritis encephalitis (CAE) or caseous lymphadenitis (CL), as carried out by GD Animal Health.

1.4.2.2. Data collection and sample size

A standardized questionnaire (appendix C) was designed to measure the exposure to putative risk factors. The questionnaire included questions on: presence of (young) cats, number of cats at farm, problems with mice/rats, water sources, use of automated mixer-feeder, storage of silage, history of outdoor access, replacement policy, and presence of other farm animals. Information on farm size and animal ages were collected from GD Animal Health database. To avoid effects from destocking and restocking during the Dutch Q fever epidemic (2009-2010), all farm management questions were referring to the past two years. During the data collection period (August 2013 to June 2014), 90 farms submitted goats' venous blood samples to GD Animal Health as part of the accreditation programs. These 90 farmers were asked to participate in this study, to which 52 agreed. The questionnaire and informed consent form were sent by e-mail or regular mail to the 52 Dutch dairy goat farms and completed by the farm owner or manager in July-August 2014. On each farm, depending on farm size, blood samples had been collected from 44 to 149 randomly selected animals of more than one year old. Available ELISA tests were sufficient for 32 goats per farm, thus, 32 frozen serum samples per farm were then selected randomly at GD Animal Health and sent to RIVM for testing.

The maximum possible within-herd prevalence with 32 sampled goats and at a confidence level of 95% was estimated using winepi.net. The minimum detectable incidence rate ratio (IR) between exposure to risk factors and infection (presence of antibodies to *T. gondii*) at herd level in this study with a sample size of 52 farms was determined based on: (1) the relative frequency of exposure among non-infected farms: 50%, (2) the ratio between non-infected and infected farms: 3, (3) a confidence level of 95% and allowable error of 10% (Noordhuizen et al., 2001).

1.4.2.3. Serological assay

Individual goat serum samples were tested with a commercial indirect enzyme-linked immune sorbent assay (ELISA) test (ID Screen Toxoplasmosis Indirect Multi-species; ID.VET Innovative Diagnostics, France) to determine the presence of *T. gondii* specific P30 (SAG1) antibodies. All steps were carried out according to the instruction of the manufacturer. Every serum sample was tested in duplicate; plate-to-plate variation of the optical density values (OD-value) was corrected by using linear regression on the control sera tested on every plate (Opsteegh et al., 2010). A plate was retested if replicates of one control had a coefficient of variation (CV) above 20% (Reed et al., 2002), or if the mean OD-value of the positive control was lower than 0.350 and/or the ratio of the mean OD-value of the positive and negative controls was lower than 3. Individual sera were retested if the replicates showed different statuses according to the cut-off values provided by the manufacturer. Information from the farm questionnaires was blinded to the laboratory technician.

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1.4.2.4. Data analysis

Information collected in the questionnaires and the serological test results were coded and entered in Microsoft Access and Microsoft Excel, and statistical analysis was performed using SPSS software version 20 and R 3.03 (IBM Corp, 2011; R Core Team, 2014).

Binary mixture analysis and estimation of seroprevalence

A suitable cut-off with corresponding estimates of diagnostic performance was obtained by fitting binary mixture models to the $\log_{10}$-transformed OD-values from the ELISA (Jacobson, 1998; Opsteegh et al., 2010). Visual inspection of the histogram of the data revealed flattened tails, thus suggesting that the components of the mixture may not be normally distributed. Therefore, pragmatically, all combinations of (1) normal and (2) shifted (along the x-axis) and optionally reflected (along the x-axis, when used for the positive component) gamma distributions were fitted and compared based on Akaike information criterion (AIC) values. Normal distributions and mixing parameter (prevalence) were fitted as described before (Opsteegh et al., 2010). For (reflected) gamma distributions, shape (α) and rate (β) parameters and a shift along the x-axis were estimated for both the negative and positive components. A cut-off value was determined at the maximum sum of sensitivity and specificity based on the final binary mixture model. Overall animal level seroprevalence was calculated based on the fraction of animals with an ELISA OD-value above the cut-off value provided by the manufacturer, and compared to the seroprevalence obtained from the binary mixture model. A farm was considered positive if at least one goat on the farm was positive in ELISA. 95% Confidence intervals for seroprevalence were based on normal approximation for the binomial distribution.

Risk factor analysis

Frequency tables of categorical variables and frequency distribution of continuous variable were constructed. Risk factors were analyzed on farm level, with mean age of the 32 selected animals per farm included as sample age. Three different modeling strategies for count data were compared: Poisson, negative binomial (NB) and zero-inflated models. Potential risk factors on farm level were analyzed by both Poisson and NB models with number of seropositive animals per farm as dependent variable and the 32 tested animals per herd as offset. In the first step, bivariable associations between each potential risk factor and the dependent variable were estimated. After testing the effect of age on seropositivity on individual animal level using a binary logistic regression model with a random herd effect, sample age was always included in the farm level models to adjust the estimates. All independent variables with p-value ≤ 0.2 were selected and the associations between those variables were tested with Chi-square test before being entered into the multivariable regression model in the second step. Decisions on inclusion of variables were made based on biological relevance, completeness of data, and the strength of relationship between the outcome and the putative risk factors. Potential confounding was determined by the change ($\geq$20%) of estimates for other independent variables before and after the factor entering the model (Dohoo et al., 2009). Missing values were excluded in the bivariable analysis, but they were coded as additional categories in the multivariable step. The model was built in a backward elimination process and two-way interaction terms of the biologically plausible variables in the multivariable model were tested. All variables with a p-value less than 0.05 in the likelihood ratio test were kept in the model. Risk was expressed as an incidence rate ratio (IR) with 95% confidence interval (Dohoo et al., 2009). The fit of the models were checked using the Pearson goodness-of-fit test and dispersion parameter. A choice was made between the Poisson model and the NB model based on the likelihood ratio test.

Finally, because of the potential presence of excess zeros in the data, a zero-inflated model was built. First, the associations between all the variables were tested with Chi-square test. All variables except correlated ones were entered into both the count and zero parts of the zero-inflated model. Next, this full model was reduced by automatic model selection based on finite sample corrected AIC

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(AICc). The process was performed using MuMIn package in R (Bartoń, 2014). The fit of the Poisson or NB model and the respective zero-inflated model was compared using a Vuong test (Vuong, 1989).

1.4.3. Results

1.4.3.1. Descriptive statistics

Epidemiological data were collected from 52 out of 90 approached farms (58%) and a total number of 1664 serum samples (32 per farm) were obtained from those farms. The participating farms originated from all provinces in the Netherlands, except Groningen and Zeeland. The mean number of goats older than one year on those 52 farms was 792 (ranging from 162 to 2083) (Fig. 2), and was not different between positive and negative farms (independent samples t-test, $p<0.05$). For 93% of the 1664 sampled animals, age information was available. Mean age of all individual animals was 3.3 years, and varied between 1 and 11.7 years (Fig. 3). The sample age per farm used in the analysis ranged from 1.3 to 7.2 years.

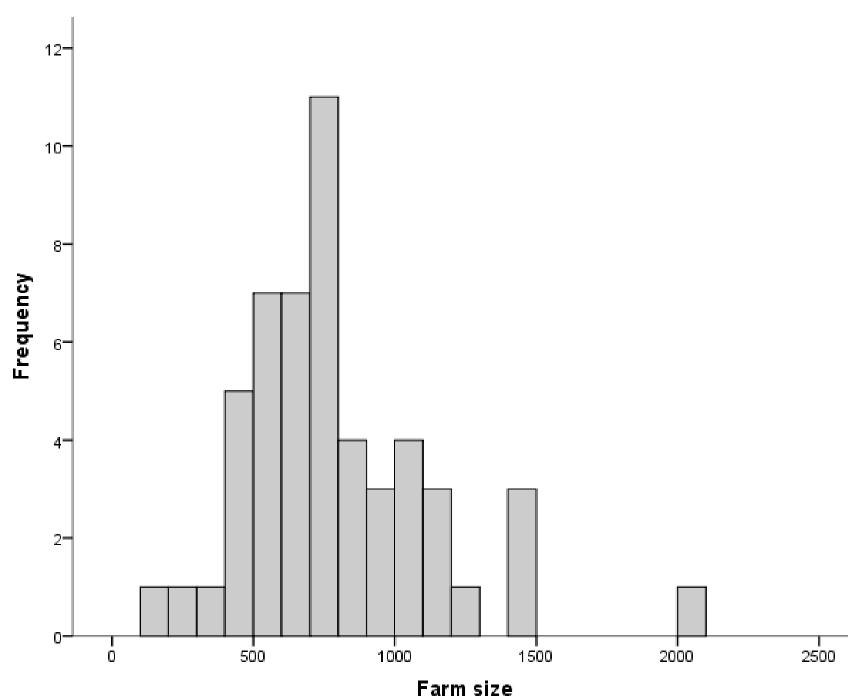

Figure 3: Frequency distribution of farm size (number of goats) for 52 participating indoor dairy goat farms.

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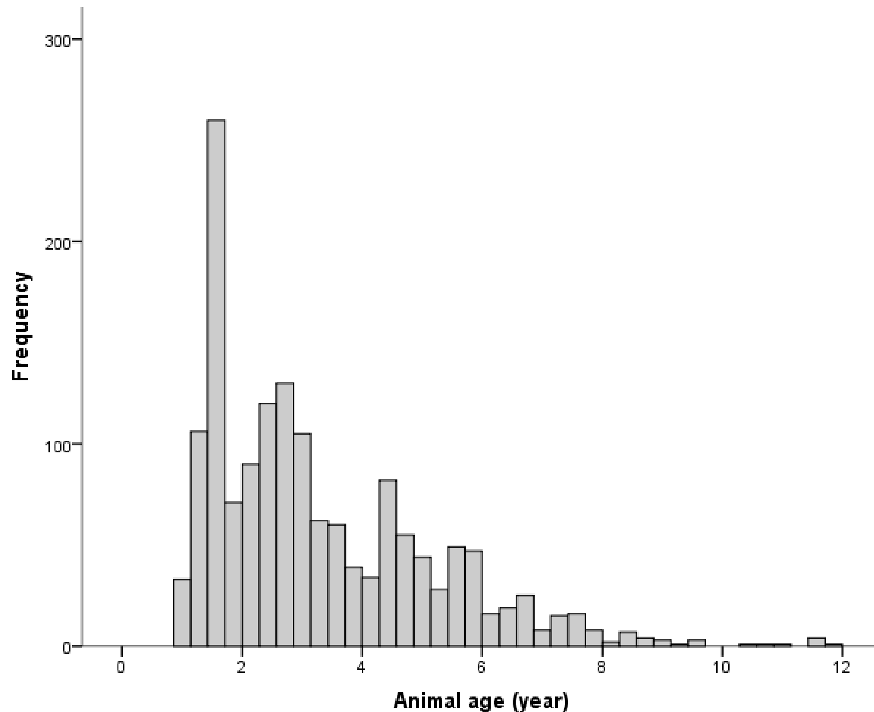

Figure 4: Frequency distribution of age (in years) for 1664 dairy goats from 52 indoor farms.

1.4.3.2. Serological results

Based on the cut-off values from the commercial test, 221 out of 1664 (13.3%, 95% CI: 11.7%-14.9%) dairy goats scored positive (S/P%≥50%, which corresponded to a corrected OD-value of 0.93) and 1443 scored negative (S/P%≤40%, which corresponded to a corrected OD-value of 0.76). Samples between 40 and 50% S/P% were defined as doubtful. On initial testing, for three animals, the duplicates showed a positive and a doubtful result. After retesting, all of the duplicates were consistently positive. Out of the 52 farms, 32 (61.5%, 95% CI: 48.3%-74.7%) had one or more seropositive dairy goat(s) present and were defined as positive farms. Among the positive farms, the prevalence of *T. gondii* infection varied from 3.1% to 96.9% (Fig. 4). The odds ratio for seropositivity of *T. gondii* increased significantly with individual age based on a binary logistic regression model with animal status as outcome variable and a random herd effect (p<0.001) (details not shown).

The frequency distribution of the $\log_{10}$-transformed corrected OD values from the ELISA test clearly showed two separated distributions. The binary model with a mixture of a shifted gamma and a shifted reflected gamma distribution had the lowest AIC value and fitted data best (Fig. 5). The negative component is described by Gamma (α=8.2, β=24.4) shifted by 0.8 along the x-axis, and the positive component is described by a reflected Gamma (α=85.2, β=10.3) shifted by 1.5 along the x-axis. The overall seroprevalence was estimated to be 13.2% based on this binary model. The optimum cut-off OD-value was 1.02 (the vertical line in Fig 5), with both sensitivity and specificity estimated at 100.0%. As there were no goats with OD-values between 0.76 and 1.02, the choice of the cut-off value (manufacturer or binary mixture model) did not affect the scoring of the goats or the estimates of seroprevalence.

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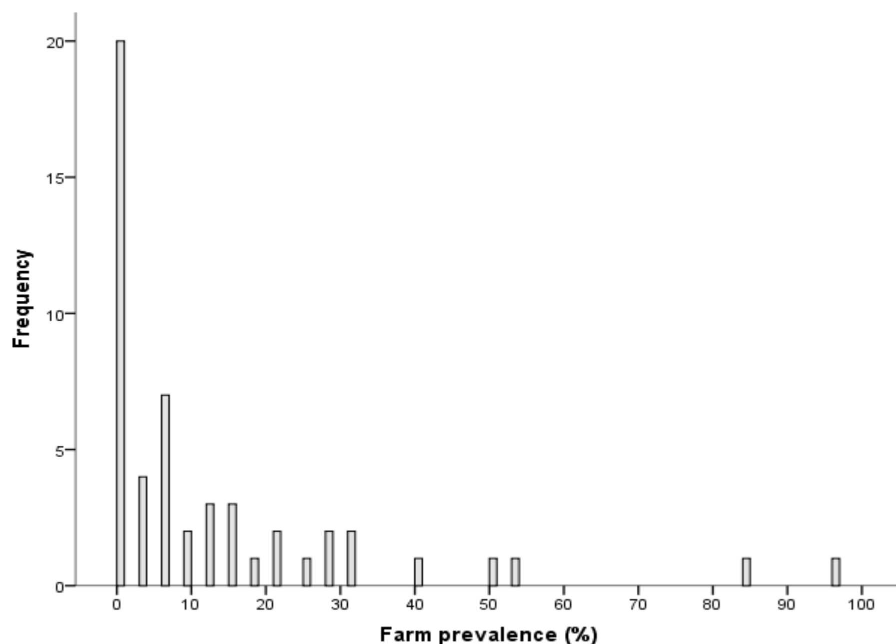

Figure 5: Frequency distribution of within farm seroprevalence of *T. gondii* in Dutch dairy goats (n=52 farms, with 32 animals tested per farm).

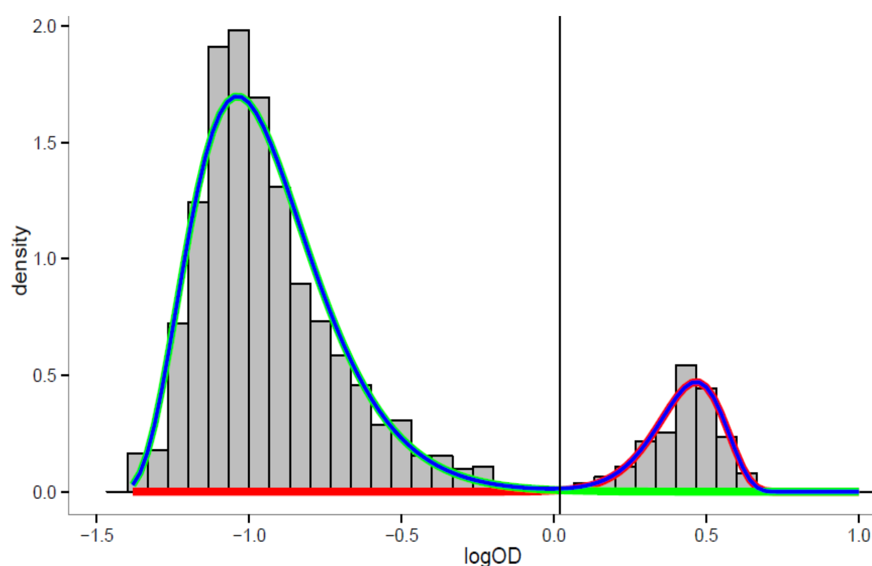

Figure 6: Frequency distribution of $\log_{10}$-transformed OD values from 1664 dairy goats in *T. gondii* ELISA (bars), fitted with a mixture (blue) of a shifted gamma (green) and a shifted reflected gamma distribution (red), and cut-off value (logOD= 0.02) (vertical line).

1.4.3.3. Risk factor analysis on herd level

The final multivariable Poisson regression model showed lack of fit (Pearson Chi-square test, $p<0.05$) and over-dispersion (Deviance/degree of freedom = 4.2, where >1 indicates over-dispersion). The likelihood ratio test suggested that the negative binomial (NB) model fitted significantly better

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than the Poisson model ($p<0.001$). In addition, a zero-inflated NB model was built, but the Vuong test indicated superior fit of the NB model ($p<0.001$). Six variables with a $p<0.2$ were found in the bivariable NB regression analysis (Table 17). However, number of cats was significantly correlated with presence of cats and presence of young cats (Pearson Chi-Square, $p<0.001$), therefore only number of cats, access of cats to the stable, use of mixer-feeder and presence of other farm animals and sample age were entered into the multivariable NB regression model. The final model maintained only two predictors: number of cats (1-4 cat: IR: 2.6, 95% CI: 1.1-6.5; >= 5 cats: IR: 14.2, 95% CI: 3.9-51.1) and sample age (IR: 1.5, 95% CI: 1.1-2.1) (Table 18). The NB model fitted the data well (Pearson Chi-square test, $p<0.05$) with a dispersion coefficient of 1.4 (95% CI: 0.8-2.5).

Table 17: Number of farms, on-farm prevalence and goat level seroprevalence of *T. gondii* by variable category, and sample age adjusted incidence rate ratio's (IR) with 95% confidence intervals and *p*-values (based on likelihood ratio test) for those variables in bivariable negative binomial regression analysis.

Variable	Category	N [a] (%)	Farm Prev. [b] (%)	Animal Prev. [c] (%)	IR	95% CI	*p*-value [d]
Presence of cats	No	14 (26.9)	50.0	4.5	Ref.		**0.016**
	Yes	38 (73.1)	65.8	16.5	3.8	1.4-10.1	
Number of cats	0	14 (26.9)	50.0	4.2	Ref.		**0.000**
	1- 4	32 (61.5)	62.5	11.7	2.6	1.1-6.5	
	>= 5	6 (11.5)	83.3	42.7	14.2	3.9-51.1	
Access of cats to goat stable	No	16 (30.8)	62.5	12.5	Ref		**0.200**
	Yes	19 (36.5)	63.2	17.9	2.1	0.7-6.7	
Presence of young cats	No	30 (57.7)	60.0	9.7	Ref.		**0.016**
	Yes	16 (30.8)	68.8	23.4	3.1	1.2-8.0	
Problems with mice or rats in the stables	No	32 (38.5)	56.2	13.2	Ref.		0.604
	Yes	20 (61.5)	70.0	13.4	1.3	0.5-3.4	
Water source	Public source only	31 (59.6)	61.3	11.1	Ref.		0.506
	Non-public source	20 (38.5)	60.0	15.3	1.4	0.6-3.3	
Use of mixer-feeder	No	26 (50.0)	50.0	10.2	Ref.		**0.122**
	Yes	26 (50.0)	73.1	16.3	2.1	0.8-5.1	
Use of silo	No, bales only	27 (51.9)	51.9	11.0	Ref.		0.262
	Yes	23 (44.2)	73.9	17.0	1.7	0.7-4.0	
Outdoor access	No	43 (82.7)	58.1	14.6	Ref.		0.337
	Yes	9 (17.3)	77.8	6.9	0.5	0.2-1.8	
Replacement policy	Closed farm	18 (34.6)	55.6	7.1	Ref.		0.240
	Only male goats introduced	24 (46.2)	62.5	18.6	2.4	0.9-6.4	
	Both male and female goats introduced	10 (19.2)	70.0	11.6	1.6	0.5-5.4	
Presence of other farm animals	No	23 (44.2)	56.5	8.0	Ref.		**0.137**
	Yes	28 (53.8)	64.3	17.0	2.0	0.8-4.9	

[a]: Total number per variable may vary because of missing values.
[b]: Farm Prev. stands for the prevalence of farms with at least one seropositive animal in each category.
[c] : Animal Prev. stands for the prevalence of seropositive animals in each category.
[d] :*p*-values ≤ 0.20 are presented bold; these factors were considered for inclusion in the multivariable model.

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Table 18: Incidence rate ratios (IR) for variables associated (p<0.05 in likelihood ratio test) with *Toxoplasma gondii* seropositivity at dairy goat farms in multivariable negative binomial regression analysis.

Variable	Category	N (%)	IR	95% CI	*p*-value
Number of cats	0	14 (26.9)	Ref.		0.000
	1- 4	32 (61.5)	2.6	1.1-6.5	
	>= 5	6 (11.5)	14.2	3.9-51.1	
Sample age	Continuous	NA	1.5	1.1-2.1	0.011

1.4.4. Discussion and conclusion

A commercial ELISA test (ID Screen Toxoplasmosis Indirect Multi-species; ID.VET Innovative Diagnostics, France) was used to determine the presence of *T. gondii* specific P30 (SAG1) antibodies in goat serum samples. This is a multispecies ELISA, with a sensitivity and specificity of 86% and 99% respectively in experimentally infected swine (Bokken et al., 2012), and a sensitivity between 95% and 97% and a specificity of 97% in Romanian household cats (Györke et al., 2011). No information on sensitivity and specificity of the assay for use with goat sera was available. Therefore, the assay was evaluated by binary mixture analysis on the frequency distribution of observed $\log_{10}$-transformed OD-values. The histogram showed two clearly separated components with an apparent right-skew for the seronegative component and left-skew for the seropositive component. When fitted with a binary mixture model, a cut-off value with sensitivity and specificity both at 100% could be found. This indicates that the serological assay has a strong discriminatory power for classifying dairy goats as positive or negative for *T. gondii* antibodies. However, external validation based on the results with a different assay would be valuable. The skewness of the distributions is confirmed by the superior fit of the combination of a shifted gamma and a shifted reflected gamma distribution and may have resulted from OD measurements outside the linear relation between OD and antibody concentration. Overall animal level seroprevalence of *T. gondii* infection was estimated at 13.3% (95% CI: 11.7%-14.9%). This seroprevalence is much lower than previously reported in goats (47%) and adult sheep (48.1%) in the Netherlands (Antonis et al., 1998; Opsteegh et al., 2010), and also low compared to results in goats in many other countries, ranging from 30.7% up to 77% (Iovu et al., 2012; Mancianti et al., 2013; Tenter et al., 2000; Tzanidakis et al., 2012). Another interesting finding was that on 38% of the 52 farms all tested goats were seronegative, whereas seropositive animals were present on all ten investigated farms in 1998 (Antonis et al., 1998). Indoor housing thus appears to reduce exposure of goats to *T. gondii* but not as much as has been shown for pigs and poultry (van der Giessen et al., 2007). This was expected as goat housing is less confined than pig or poultry housing, with e.g. natural ventilation and bedding, silage and roughage introduced into the stable.

A short standardized questionnaire was used for farm level risk factors. Potential risk factors without anticipated variation between Dutch herds, i.e. the bedding material, type of housing and feeding system, were excluded from the questionnaire. To evaluate the potential risk factors, three modeling strategies were compared. The dataset was clustered and the outcome variable showed over-dispersion (Fig. 4). Therefore, a NB model was built, and the dispersion coefficient in the NB model was significantly larger than zero, indicating this model was more appropriate than a Poisson model. To evaluate the presence of access zeros, a zero-inflated NB model was additionally built, but the Vuong test indicated that the NB model fitted better. Number of cats remained a significant predictor in the NB model for the seroprevalence of goats in the farm, with an increased positive association for farms with 1-4 cats to farms that had ≥ 5 cats. This finding is consistent with previous reports (Cavalcante et al., 2008; Neto et al., 2008). Felids are the only known definitive host of *T. gondii,* and primary infected cats shed millions of oocysts in the environment (Tenter et al., 2000). The association

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with the presence of cats is therefore assumed to indicate a causal relationship, and limiting the number of cats at goat farms is expected to reduce *T. gondii* infections in goats. In this study, other variables that could potentially increase exposure to cat shed oocysts (e.g. access of cats to the stable, water source, use of silo, use of mixer-feeder, and history of outdoor access) did not show a statistically significant association with *T. gondii* seropositivity. This may have been due to a lack of power. Fifty-two farms participated and 32 animals per farm were tested. With 32 animals tested per farm of 792 goats on average, farms can be misclassified as negative and the maximum possible prevalence at negative farms is 8.8%. This type of misclassification is unlikely to depend on the exposure to risk factors, but can reduce the risk estimates. In addition, with 52 farms, the minimum detectable IR between exposure to risk factors and presence of toxoplasmosis at herd level is estimated at 4, indicating that predictors with a weaker effect were unlikely to be identified as statistically significant in this study.

In conclusion, the serological assay used in this study was suitable for the detection of *T. gondii* antibodies in dairy goats. Indoor-housed Dutch dairy goats were not free from *T. gondii* infection, but the seroprevalence of these indoor kept dairy goats was lower than the published findings in outdoor goats in other European countries. Moreover, the number of cats at the farm was clearly associated with the number of seropositive goats. Since the overall animal level seroprevalence was 13.3% and a positive relationship between detection of antibodies against *T. gondii* and presence of tissue cysts in meat was found in this species, goats could be a source of *T. gondii* infection for humans. Goat meat and milk should be given a sufficient heat treatment to kill the parasites before consumption. Limiting the presence of cats at the goat farms is expected to reduce the prevalence of *T. gondii* infected goats.

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2. Experimental studies in pigs

Three studies were performed in pigs and will be described in this chapter. First, the correlation between the presence of anti-*T. gondii* IgG (infection) and presence of tissue cysts in pigs was studied in France (2.1). Second, the anatomical distribution of tissue cysts in different cuts of meat intended for the food chain following infection with two infective stages of the parasite (oocysts and tissue cysts - bradyzoites) was studied in pigs by means of MC-PCR. The effect of vaccination to prevent tissue cyst formation using S48 was also studied (2.2). Third, a study to analyse risk factors for *T. gondii* infection in pigs in the UK was performed (2.3).

2.1. The relationship between serology, bioassay and PCR based detection of *Toxoplasma gondii* in a slaughterhouse study in pigs in France

2.1.1. Introduction

Pork is often considered to be a major source of *T. gondii* infection, e.g. in the USA pork and *T. gondii* are ranked second among the top 10 pathogen-food combinations in terms of annual disease burden (Batz et al., 2012). The data available from the literature review, suggests that there is a positive relationship between detection of antibodies in sera and presence of viable tissue cysts of *T.gondii* in pigs. However these data are based on a large majority of articles (17/18) using as direct detection methods the (mouse or cat) bioassays while direct detection using PCR-based methods appears to be rarely performed (1/18). In addition, there is an indication that direct detection by (mouse or cat) bioassay might be possible also from seronegative animals.

For this purpose, we used the sampling plan that was organised at a nationwide scale and financed by the French Ministry of Agriculture, in 2013. The sampling strategy was established with the objective of being representative of pigs slaughtered in France. The sampling was conducted in 6 administrative regions that account for 90% of the French pig production. The regions have been identified at a national level with the help of BD-Porc (national pig farms database). The numbers of samples collected during this study are shown in the Table 1. They represent 97.3% of the planned samples, with a high discrepancy for piglets and sows from the outdoor housing system.

Table 1: Number of samples collected in France during the course of 2013, according to the housing system and age

FRANCE	Indoor	Outdoor	Total
Piglets	126	5	131
Fattening)	963	197	1158
Sows	251	7	258
Total	1340	209	1549

To study the relationship between bioassay, qPCR, MC-PCR and serology in pigs, serum, a 200 g tissue sample from the heart, considered as a predilection tissue and a 100 g sample from an edible tissue (diaphragm) were collected from 1549 pigs in France. Information on age, sex, type of farming (indoor, outdoor), and sampling date was obtained. Serum and tissue fluid samples from the heart were tested to detect anti *T. gondii* IgG by modified agglutination test (MAT). The sample from the heart was tested by mouse bioassay (200 g using a harmonised protocol for artificial ingestion, and by inoculation of 2 mice per sample) and a fraction of the digest was tested by qPCR.

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Aim of the study:
To assess the concordance between serology, bioassay and PCR-based detection, within the EFSA project. The edible tissue by means of serology (MAT on tissue fluid) and MC-PCR based molecular diagnostics were analysed while sharing with EFSA the previous results (bioassay, qPCR on heart tissue).

2.1.2. Material and Methods

2.1.2.1. Sample collection

Out of all the samples from 1549 pigs collected, the mouse bioassay was performed for 160 heart samples (69 samples from MAT positive pigs cut-off value >1:6 and 91 samples from randomly selected seronegative pigs) and these 160 samples were further analysed in the present project.

2.1.2.2. Antibody detection in pigs

All collected pig sera, diaphragms and cardiac fluids were sent to ANSES – USC EpiToxo in Reims and tested by a modified agglutination test (MAT) according to appendix 6.1.3. The results are available in Table 3 of section 2.1.3.1.

2.1.2.3. Mouse bioassay

Mouse bioassay was performed as described (appendix 6.1.1) with slight modifications: mice were bled after 6 weeks from the retro orbital sinus and tested by MAT for *T. gondii* IgG antibodies. Only seropositive mice cut-off value ≥1:6 in contrast to all mice in cattle and horse study, were euthanized and brains were collected aseptically. Half of the brain of each mouse was collected for qPCR and RFLP genotyping, while the other half was sent to ANSES, Reims for strain collection and preservation.

2.1.2.4. PCR on heart digest

The qPCR on heart digest used the same target 529bp RE, but with a slight different protocol than the one in the appendix. Briefly, the DNA extraction was performed on 300µl of sample, with a final volume of DNA extract of 100µl. PCR amplification was performed in 96-wells plates using a LightCycler480 thermal-cycler instrument (Roche, Almere, Netherlands). The 20 µl reaction mixture consisted of 12.5 µl Platinium-UDG PCR super mix (Eurofins), 0.5 µM of each primer (HO1 and HO2), 0.2 µM of HOFT probe (Table 2), 5 µl double distilled water, and 5 µl of template DNA. Each sample was tested in triplicate. The reaction mixture was initially incubated at 50°C for 3 min, and then 95°C for 3.5 min to activate DNA polymerase. Initial incubation was followed by 40 amplification cycles that consisted of a denaturation step at 95°C for 15 s, and annealing step at 60°C for 60s. Afterwards, the samples were cooled at 37 °C for 1 min. The temperature transition rate was 4.40°C/s for increasing temperatures and 2.20 °C/s for decreasing temperatures. Fluorescence at 530 nm (HOFT) was measured at the end of each extension step. Sample was considered PCR positive when Cq values ≤35 in at least 2 out of 3 replicates.

Table 2: Oligonucleotide sequences of primers and probe used for qPCR in pig slaughterhouse study

Name	Sequence 5'-3'	5'label	3'label
HO1	AGA GAC ACC GGA ATG CGA TCT (21)	-	-
HO2	CCC TCT TCT CCA CTC TTC AAT TCT (24)	-	-
HOFT	ACG CTT TCC TCG TGA TGG GG (20)	FAM	TAM

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2.1.2.5. MC-PCR

MC-PCR was performed at ENVA on the frozen edible tissue samples (diaphragms) for all 160 pigs irrespective of their serological result. MC-PCR was performed as described in appendix 6.1. PCR was considered positive using the criteria described for PCR on mouse brain (section 1.1.2.3).

2.1.2.6. Data analysis

All statistical analyses were done in the R Core Team (2013). R is a language and environment for statistical computing made by R Foundation for Statistical Computing, Vienna, Austria (http://www.R-project.org/). To calculate agreement values with 95% confidence intervals Cohen's kappa test was used (fmsb package). Six different concordance categories as defined in appendix B were used for interpretation.

To evaluate concordance between MAT results on different matrices (serum, cardiac fluid and diaphragm fluid) Cohen's Kappa values were calculated. Kappa concordance was also calculated to compare the presence of IgG antibodies as determined by MAT with direct detection of parasites in pig tissues. For this comparison mouse bioassay, PCR on heart digest and MC-PCR on diaphragm are considered separately and as a combined variable of any direct detection positive result.

2.1.3. Results

2.1.3.1. Detection of IgG antibodies against *T. gondii* in pigs

Blood was collected in the slaughterhouses from 118 of the 160 pigs and diaphragm fluids of 88 pigs could be collected in the laboratory, while we could collect 160 cardiac fluids. The IgG detection by MAT on 3 diferent matrices is summarized in Table 3 on these 3 different matrices.

Table 3: Modified agglutination test (MAT) titers and classification (cut-off value ≥1:6) on three different matrices (serum, cardiac fluid, and diaphragm fluid)

MAT	Serum	Cardiac fluid	Diaphragm fluid
negative	46	91	43
positive	72	69	45
1:6	17	15	9
1:12	9	12	3
1:25	7	11	11
1:50	8	13	8
1:100	13	6	8
1:200	8	4	5
1>200	10	8	1
Total	118	160	88

For 20 pigs, the serum samples tested negative while the corresponding CF or/and DF samples were positive using MAT. Since it is biologically not plausible that antibody levels are higher in CF and/or DF than in serum, a mismatching of samples most likely occurred. Therefore, these samples were excluded from further analysis.

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The concordance analysis has been performed pairwise for: sera vs cardiac fluid: 98 samples; sera vs diaphragm fluid: 51 samples; and cardiac fluid vs diaphragm fluid: 80 samples. The concordance of MAT results performed on the three different matrices was fair for each pair analysed (Table 4).

Table 4: The concordance of MAT results performed on the three different matrices

MAT performed on:	Cohen's kappa	95%CI
sera vs cardiac fluid	0.35 (fair)	0.18 - 0.52
sera vs diaphragm fluid	0.30 (fair)	0.03 - 0.57
cardiac fluid vs diaphragm fluid	0.21 (fair)	-0.002 - 0.42

2.1.3.2. Direct detection of *T. gondii* in pigs

Out of 160 samples selected for parasite isolation, 160 were analysed by mouse bioassay, 159 by qPCR on heart digest and all 160 by MC-PCR on diaphragm.

The presence of *T. gondii* was demonstrated in the tissues of 82 out of 160 pig samples (Table 5). As the 160 samples have been selected based on the serology result, a statistical evaluation of positivity detected by direct tests by age, sex or type would be highly biased. Positive results were obtained for one, two or all three techniques (Fig. 1). A description of the positive animals (14) with an overlap in positive results from mouse bioassay, qPCR on heart digest and MC-PCR on diaphragm is provided in Table 6.

Table 5: Detection of *T. gondii* in the tissues of pig by direct detection techniques

	N° of positive results/n° of analysed samples
Mouse bioassay	41/160
PCR Heart Digest	69/159
MC-PCR	27/160
Any of the 3 techniques	82/160

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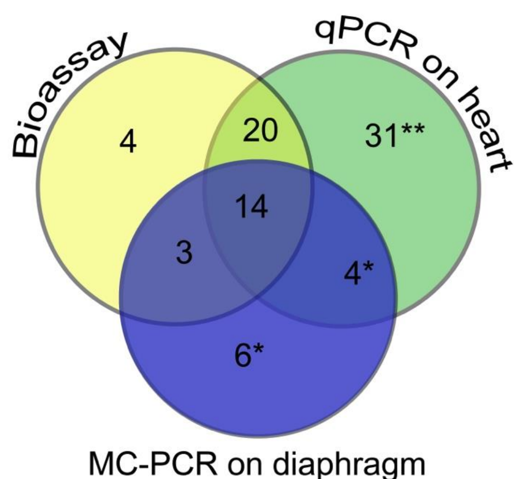

Figure 1: Total number of positive results obtained by direct detection methods in pigs. Numbers correspond to the numbers of animals found positive at one, two or three methods. * or ** corresponds to one or two mice that died in the first 2-3 days p.i. so no result for bioassay was taken into account.

Table 6: Details for the 14 pigs which tested positive for all three direct detection methods

Animal ID	Age category	Production type	Indirect detection methods			Direct detection methods		
			MAT Sera	MAT Cardiac fluid	MAT Diaphragm	MC-PCR	Bioassay in mice	qPCR
P0146	Fattening	Outdoor	1/200	1/50	1/50	37.83	POS	33.87/33.30
P0161	Fattening	Outdoor	1/100	1/50	1/200	31.66	POS	31.79/30.89
P0164	Sow	Indoor	1/100	1/200	1/100	38.33	POS	33.22/39.39
P0197	Fattening	Indoor	No sera	1/25	1/50	38.33	POS	32.39/32.61
P0515	Fattening	Outdoor	>1/200	1/200	No fluid	26.81	POS	33.57/31.39
P0517	Fattening	Outdoor	>1/200	>1/200	No fluid	34.00	POS	27.83/27.88
P0520	Fattening	Outdoor	>1/200	>1/200	No fluid	26.47	POS	30.94/30.38
P0522	Fattening	Outdoor	0	1/100	0	29.19	POS	32.90/32.04
P1112	Fattening	Indoor	100	1/50	No fluid	30.57	POS	25.6/24.8/24.9
P1179	Sow	Outdoor	100	1/50	No fluid	34.87	POS	29.0/28.4/29.5
P1298	Fattening	Outdoor	100	1/25	No fluid	26.74	POS	27.1/26.8/27.3
P1410	Fattening	Outdoor	400	1/100	1/100	38.33	POS	22.52/22.10/22.83
P1481	Sow	Indoor	200	1/100	1/50	38.33	POS	29.31/0/0
P1499	Fattening	Outdoor	No sera	1/25	No fluid	37.35	POS	27.48/26.09/26.34

Mouse bioassay: Twenty-two mice (originating from 11 pigs) died within 2-3 days after inoculation, but in none of these mice clinical toxoplasmosis was suspected or demonstrated (toxic effects

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suspected) and one bioassay (2 mice) needed to be cancelled due to experimental errors. The presence of anti *T. gondii* IgG was demonstrated in the tissues of 41 out of 148 remaining pigs (27.7%), by MAT on mice sera and qPCR on seropositive mouse brain.

qPCR heart digests: Out of the 159 digests tested, 69 (43.4%) had a positive amplification of *T.gondii* specific 529bp RE.

MC-PCR: Magnetic capture of the *T. gondii* 529bp RE was performed on DNA extracted from diaphragms of 160 pigs. Twenty-seven (16.9%) of them were considered positive.

2.1.3.3. Concordance between presence of IgG and detection of *T. gondii* in tissues

The direct detection results are presented by serological status in Tables 7-9. A MAT titer of ≥1:6 was considered positive. First the MAT results on various matrices (cardiac fluid, serum and diaphragm fluid) were compared to bioassay (Table 7) and to any direct detection method (mouse bioassay or PCR on heart digest or MC-PCR on diaphragm) (Table 8).

Table 7: Concordance between detection of antibodies by MAT on three matrices (cardiac fluid (CF), sera and diaphragm fluid (DF)) and demonstration of viable *T. gondii* in heart by mouse bioassay

	Bioassay			Recovery	Cohen's kappa	
MAT CF	negative	positive	total	rate	Estimation	95%CI
negative	78	5	83	0.06	0.66	0.52-0.80
positive	14	32	46	0.69	Substantial	
MAT Sera						
negative	23	1	24	0.04	0.28	0.10-0.45
positive	37	31	68	0.45	Fair	
MAT DF						
negative	29	6	35	0.17	0.20	-0.02-0.42
positive	23	14	37	0.38	Poor	

Table 8: Concordance between detection of antibodies by MAT on three matrices (cardiac fluid (CF), sera and diaphragm fluid (DF)) and any direct detection of *T. gondii* (mouse bioassay, PCR on heart digest or MC-PCR on diaphragm)

MAT CF	**Any direct detection method**			Recovery	Cohen's kappa	
	negative	positive	total	rate	Estimation	95%CI
negative	54	29	83	0.35	0.41	0.26-0.58
positive	9	37	46	0.80	Moderate	
MAT Sera						
negative	15	9	24	0.37	0.18	-0.02-0.39
positive	27	41	68	0.60	Poor	
MAT DF						
negative	22	13	35	0.37	0.22	-0.01-0.45
positive	15	22	37	0.59	Fair	

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As shown in the tables 7 and 8, the highest Cohen's kappa values were detected between the MAT cardiac fluid and the bioassay or any direct detection method (0.66 and 0.41, respectively). This result, alongside with the suspicion of mismatching the collected samples (see above in the material and method section), enable us to consider forfurther analysis the cardiac fluid and MAT CF as the "reference" matrix respectively the "reference" result to be compared with.

Table 9: Concordance between detection of antibodies by MAT on cardiac fluid (CF) and PCR-based detection of *T. gondii* DNA in heart (qPCR) and diaphragm (MC-PCR)

MAT CF	**qPCR** negative	positive	total	Recovery rate	Cohen's kappa Estimation	95%CI
negative	67	21	88	0.24	0.46	0.31-0.61
positive	15	37	52	0.71	Moderate	
MAT CF	**MC-PCR**					
negative	79	9	88	0.10	0.23	0.05-0.42
positive	36	16	52	0.31	Fair	

MAT CF has the best concordance with mouse bioassay (0.66) (Table 7) compared to other direct detection techniques (qPCR on heart digest: 0.46 or MC-PCR on diaphragm: 0.23) (Table 9).

2.1.3.4. Concordance between direct detection methods of *T. gondii* in pig tissues (mouse bioassay, PCR on heart digest and MC-PCR on diaphragm)

The concordance of the three direct detection methods are compared in Tables 10 and 11. The best concordance is noticed for mouse bioassay and qPCR on heart digest (0.50), while the 2 PCR based techniques (PCR on heart digest and MC-PCR on diaphragm) showed fair concordance (0.21).

Table 10: Concordance between direct detection of *T. gondii* in pig by mousebioassay and PCR-based detection of *T. gondii* DNA in heart (qPCR) and diaphragm (MC-PCR)

qPCR	**Bioassay** negative	positive	total	Recovery rate	Cohen's kappa Estimation	95%CI
negative	68	6	74	0.08	0.50	0.35-0.66
positive	24	31	55	0.56	Moderate	
MC-PCR						
negative	84	22	106	0.21	0.36	0.16-0.56
positive	8	15	23	0.65	Fair	

Table 11: Concordance between PCR-based detection of *T. gondii* DNA in trypsin-digest of heart (qPCR) and detection of *T. gondii* DNA in 100g of diaphragm by magnetic capture and qPCR

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MC-PCR	qPCR			Recovery rate	Cohen's kappa	
	negative	positive	total		Estimation	95%CI
Negative	74	41	115	0.36	0.21 Fair	0.04-0.39
Positive	8	17	25	0.68		

2.1.4. Discussion and conclusion

In 2013, a nationwide study was conducted in France, with the help of Ministry of Agriculture, aiming to investigate the *T. gondii* prevalence in pork produced in France. A total of 1549 hearts, 1520 diaphragms and 1066 blood and cardiac samples were tested using the Modified Agglutination Test (MAT). An overall seroprevalence of 3% in indoor pigs and 6.3% in outdoor pigs was obtained based on MAT on cardiac fluid. A total of 160 heart samples, corresponding to all positive MAT (sera or cardiac fluids) samples and randomly selected negatives, were tested by mouse bioassay and qPCR. In addition, the corresponding diaphragms (160) were analysed under the present EFSA project using MAT on diaphragm fluid and MC-PCR on diaphragm tissue. The results were compared with the previous data (MAT on sera, MAT on cardiac fluid, mouse bioassay and qPCR on heart digest).

Results of the MAT on all cardiac fluids of 160 selected pigs and on a limited number of sera of 118 pigs and on diaphragm fluid of 88 pigs were analysed. The PCR based techniques (qPCR on heart digest and MC-PCR on diaphragm) were performed on all samples except one qPCR analysis that was lacking. A total of 22 mice, originating from 11 pigs, died within the first 2-3 days after inoculation in the mouse bioassay, but in none of these mice clinical toxoplasmosis was suspected nor demonstrated by PCR on intraperitoneal fluid or re-inoculation of the original heart digest. Since the inoculated samples were originating from all three age categories (piglets, fattening, sows), age-related toxic effects (like in cattle study) can be excluded and might be caused by a bacterial contamination of the inoculum causing death. Adding of an alternative antibiotic cocktail (Vancomycine®, Ciprofloxacine®, Cefotaxim®) during the heart digestion technique, we were able to reduce the post-inoculation mortality completely.

According to the literature review, MAT showed higher sensitivity than ELISA or IFAT, on pig sera and tissue fluids. Here, we had chosen the very first dilution (1:6), as a cut-off for all matrices (sera, cardiac fluid, and diaphragm fluid) to study the concordance of IgG detection and presence of viable parasites. Out of the 160 samples, antibodies were detected by MAT in 61% of pigs according to the sera, in 43% according to the cardiac fluid and in 51% according to the diaphragm fluid (Table 5). However when the results obtained from MAT (sera, cardiac fluid and diaphragm fluid), were analysed according to the Cohen's kappa test, the concordance amongst them was fair for each pair but not more than that. The absence of a higher concordance may be explained by several hypotheses:

1. The quality of the matrix to be analysed is not the same in terms of

    a. concentration of circulating IgG: the serum is the tissue with the highest level of IgG, followed by cardiac and diaphragm fluid. The serum was obtained following blood collection from jugular vein. The cardiac fluid was collected from the plastic container used for heart transport, being a mix of serum, expressed from the existing blood clot in the heart and tissue fluid. The diaphragm fluid was obtained by a thawing process, containing though an important quantity of water and very low quantity of blood/sera. This hypothesis is strongly supported by the concordance diagonal calculated based on our results (figure 4), showing a

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decrease in antibody titer in CF when compared to sera. Therefore, a large proportion of the discordant results might have been caused a low sensitivity of MAT in case of CF and DF eventually caused by a lower antibody concentration in fluids relative to serum.

b. uniformity/homogeneity of cardiac and diaphragm fluid collection from one sample to another, to a lesser extent also applicable for sera.

2. A lack of MAT specificity by using the 1/6 cut-off. By using a higher cut-off (1/12 or 1/25) higher concordance for the different matrices was observed (data not shown). However, when using a higher cut-off value (≥1/25), 5 bioassay positive strains would have been assigned to pigs with serological results below the threshold limit (2 isolated strains from pigs with a 1/6 titer and 3 isolated strains from pigs with 1/12 titer).

Moreover, the samples were collected within the slaughterhouses by the local veterinary technicians. Therefore a mismatch of samples (blood vs heart + diaphragm), could have occurred during the collection in the slaughterhouse, leading to an inversion in positive/negative results, leading to false negatives/positives. As a matter of fact, 20 samples presented a CF or/and DF positive analysis while the corresponding sera was negative. Within a normal immunological answer, the first body-fluid to become positive for antibodies detection should be the sera, followed by the other body-fluids. Therefore the mismatching hypothesis along side with a coherent biological answer from the host is the basis for excluding the 20 samples with results which are not plausible.

When we compared the MAT technique performed on three different matrices (sera, cardiac and diaphragm fluid) with the direct detection methods (bioassay, qPCR and MC-PCR), the highest Cohen's kappa values are observed for MAT cardiac fluid (0.66 with bioassay and 0.41 with any direct detection method). Based on these results and the "mismatching hypothesis" (see above) the MAT on cardiac fluid is considered the "reference" matrix, which we used for further analysis.

Analysing the direct detection methods (mouse bioassay, qPCR on heart and MC-PCR on diaphgram) results, qPCR on heart digest was the most sensitive method with 69 (43.1%) positive results, followed by bioassay with 41 (27.7%) and MC-PCR with 27 (16.9%) positive results. These differences in sensitivity might be explained by a) the use of the predilection site (heart) for qPCR and bioassay vs MC-PCR (diaphragm); b) the presence of viable or dead parasites (PCRs vs bioassay) combined with c) the low parasite burden. When we correlate the three direct detection methods the best concordance that we found is between qPCR and bioassay (0.50) compared to MC-PCR and bioassay (0.36) and between the 2 PCR techniques: qPCR and MC-PCR (0.21).

The concordance between qPCR and mouse bioassay might be explained by: a) the use of the same matrix for the analysis (predilection site=heart); b) the digestion method of the heart, prior to DNA extraction or mouse inoculation, that helped liberation of bradyzoites from the cysts, resulting in more homogenous number of parasites per µl of the sample and c) the performance of the qPCR only on serologically positive mice, increasing though the chances of positive results (not the case in cattle and horse study, where qPCR has been performed on all mice). The lack of a higher concordance between the 2 techniques can be explained by: a) a difference in the quantity of the sample used for analysis; b) the parasite burden: the time frame from pig infection to the slaughtering it might be short (several days), not letting the parasite to replicate itself in higher numbers; c) different target detection (viable parasites vs parasitic DNA/dead parasites).

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The concordances between MC-PCR and mouse bioassay and qPCR on heart digest were fair (Cohen's kappa 0.36 and 0.21, respectively), lower than for the previous analysed couple. This discrepancy might be explained first of all by: a) the use of a different matrix (the diaphragm) than the predilection site=heart; b) the use of a matrix which was frozen for more than one year, storage condition that might contribute to the DNA destruction; c) the use of different DNA extraction protocols.

The overall direct detection rate, based on any positive direct detection test, in antibody detection positive animals (80.43%) is significantly higher than the detection rate in antibody detection negatives (34.91%) (Pearson's χ^2, *p*-value<0.001). At a closer look, the overall direct detection rate, based on mousebioassay only, is still significantly different in seropositive animals (69,6%) compared to seronegatives (6%) (Pearson's χ^2, *p*-value<0.001). This result is comparable with the results analyzing the literature, where a detection by direct assays (cat- or mouse bioassay mainly) was 58.8% in seropositives. However, the relatively high percentage of overall (any) direct detection rate in seronegative animals (34,91%) is different from the literature where we reported 4.9% in seronegatives. One possible explanation might be the use of relatively low number of inputs from articles using PCR based methods within the literature review (only 1 article). However, this discrepancy needs further investigation. One strain was isolated by mouse bioassay from one animal that was negative in both indirect detection methods (MAT on sera and cardiac fluid). The PCR detection methods had a positive amplification for MC-PCR on diaphragm and a negative result for heart digest. This result might be explained by a) the use of different digestion techniques: proteinase K digestion of diaphragm and trypsin digestion of the heart; b) the use of different DNA extraction techniques: magnetic capture of DNA for diaphragm and column purification of the DNA for heart; c) the use of different PCR amplification protocols; d) the mismatching hypothesis of the heart and diaphragm.

Conclusions:

1. There is a low concordance amongst the three matrices (sera, cardiac fluid and diaphragm fluid) tested for the detection of circulating antibodies with the same technique (MAT) (0.35; 0.31; 0.20).

2. Diaphragm fluid showed the lowest concentration of circulating *T.gondii* specific antibodies detected by MAT.

3. The antibody detection and the presence of parasites are positively correlated. However sera represent a good matrix for epidemiological survey while cardiac fluid has the best concordance with mouse bioassay representing though the “reference” matrix for viable parasites isolation

4. The overall direct detection rate in antibody detection positive animals (80.43%) is significantly higher than the detection rate in antibody detection negative animals. However, the relatively high detection rate of *T. gondii* in seronegatives (34% as found in this study) need further investigation

One strain was isolated by mouse bioassay from one animal that was negative both by indirect detection methods (MAT on sera and cardiac fluid) and direct detection methods (qPCR on heart digest), indicating the potential of a human risk for infection also from seronegative pigs.

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2.2. *Toxoplasma gondii* vaccination and challenge experiment in pigs

2.2.1. Introduction

The vaccine to prevent *T. gondii* abortions in sheep (Toxovax) can possibly also prevent tissue cyst formation. In that case, vaccination of food animals other than sheep can be valuable for the prevention of human toxoplasmosis. Therefore, experimental infections by oral inoculation with tissue cysts or oocysts in unvaccinated and Toxovax-vaccinated pigs have been carried out at Moredun Research Institute (Burrells et al., 2015). Mouse bioassay and ITS1 nested PCR had already been completed for this study in brain, a pooled sample of heart, tongue, masseter and diaphragm, and a pooled sample of chop, loin, left hind limb, and left forelimb and results are available for comparison, within the present EFSA project, between bioassay and MC-PCR.

Aim of the study

This experiment was aimed to determine by means of MC-PCR the dissemination of parasite tissue cysts to different tissues, organs and cuts of meat intended for the food chain (chop, loin, left forelimb, left hindlimb), following infection with two infective stages of the parasite (oocysts and tissue cysts - bradyzoites). The effect of vaccination to prevent tissue cyst formation using S48 was also studied.

The following hypotheses were tested:

(A) Pigs become infected with *T. gondii* by oral inoculation with both oocysts and bradyzoites.

(B) Tissue cyst formation by both stages of the parasite can be detected within tissues intended for human consumption, a potential risk to humans.

(C) Vaccination reduces the number of tissue cysts or prevent tissue cyst formation, and therefore help to produce safer pork.

2.2.2. Material and Methods

2.2.2.1. Study design

This experiment was completed at Moredun Research Institute in 2011. Twenty-three large white landrace pigs were assigned to experimental groups described in Table 12.

Table 12: Grouping of pigs in *Toxoplasma gondii* vaccination and challenge experiment

Group	n	Treatment	Frequency of treatments	Purpose
1	5	Infection with 1000 M4 *T. gondii* tissue cysts. Animals culled 6 weeks post infection.	1	To establish infection with *T. gondii* and to confirm tissue cyst ingestion leads to infection. To determine which tissues the parasite will establish in and which are best for parasite detection.
2	5	Infection with 1000 sporulated M4 *T. gondii* oocysts. Animals culled at 6 weeks post infection	1	To establish infection with *T. gondii*. This group will allow study of cyst formation within different tissues following infection with oocysts, and to generate control material for future work.

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Group	n	Treatment	Frequency of treatments	Purpose
3	5	Vaccination with commercial vaccine (1.2x10^5 S48 tachyzoites) 4 weeks before infection with 1000 sporulated M4 *T. gondii* oocysts. Animals culled at 6 weeks post infection.	2	To evaluate cyst formation with the ability of the vaccine to prevent cyst development being studied.
4	5	Vaccination without challenge (1.2x10^5 S48 tachyzoites). Animals culled at 6 weeks post infection	1	Control group. Vaccination should not cause any pathology or tissue cysts. If infected, molecular techniques can distinguish vaccine strain from local strains.
5	3	Control animals inoculated with the dilution media. Animals culled at 6 weeks post infection	1	Control group with no infection and no vaccination, acting as biological control for immunology.

For this project, only the animals from groups 1, 2 and 3 were tested.

2.2.2.2. Sequence-based magnetic capture and 529 bp RE qPCR

Sequence-based magnetic capture was performed on pools of tissue, with 10g tissue of 5 animals per pool. MC-PCR is described in appendix 6.1. Lysis buffer was scaled down for 50g samples.

2.2.3. Results

All pigs in group 1, 2 and 3 were seropositive by ELISA by day 21 (for further details refer to (Burrells et al., 2015)). Mouse bioassay and MC-PCR results are presented in Table 13. Only one individual porcine tissue was positive by ITS1 PCR, chop from animal 830 (group 1, tissue cyst challenged pigs).

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Table 13: Molecular detection of *T. gondii* from porcine tissues and mouse bioassay

			Mouse Bioassay									Tissue: MC-qPCR cp value			
			Mouse 1			Mouse 2			Mouse 3						
Treatment	Group	Animal	Cull date (dpi)	ITS1 PCR (Food)	ELISA Result	Cull date (dpi)	ITS1 PCR (Food)	ELISA Result	Cull date (dpi)	ITS1 PCR (Food)	ELISA Result	*Longissimus dorsi* (chop)	*Psoas major* (loin)	Left *Tricep femoralis* (forelimb)	Left *Semitendinosus* (hindlimb)
Tissue cyst challenged	1	830*	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		831	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		832	42	Positive	Neg	42	Neg	Neg	42	Neg	Neg	35.32	34.04	35.56	0
		833	42	Neg	Neg	42	Neg	Neg	11	Positive	Neg				
		834	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
Oocyst challenged	2	825	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		826	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		827	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg	33.70	33.61	0	0
		828	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		829*	12	Positive	Neg	12	Positive	Neg	12	Neg	Neg				
Vaccinated and oocyst challenged	3	820	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		821	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		822	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg	0	0	0	0
		823	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				
		824	42	Neg	Neg	42	Neg	Neg	42	Neg	Neg				

830* = ITS1 positive from inocula (2/3 replicates); 830* = ITS1 positive from inocula (3/3 replicates). Food group pool was comprised of chop, loin, forelimb and hindlimb. Mouse bioassay results are described in Burrells *et al* 2015. Tissue sample = 10g each - 50g Total, pool of 5 animals per tissue per group.

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2.2.4. Discussion and conclusion

The results from MC-PCR completed as part of this EFSA project correlated well with the mouse bioassay and ITS1 PCR results which had been completed previously (Table 13) and (Burrells et al., 2015). The high MC-PCR Cq values from tissues tested within groups 1 and 2 indicate low parasite burden within the positive tissues (chop and loin). This is reassuring as pigs were challenged with 1000 oocysts, therefore, parasite burden is likely to be lower than that described for lambs (challenged with 500,000 oocysts).

Using MC-PCR the parasite could be detected within chop and loin from the non-vaccinated oocyst challenge group (group 2) and chop, loin and forelimb from the non-vaccinated tissue cysts challenge group. Cq values did not vary much (33.61 – 35.56) between the tissues or within infection groups.

Finally, the parasite was not detected by MC-PCR or ITS1 PCR in the vaccinated and challenged group, therefore the vaccine does not appear to induce tissue cysts and reduces tissues cyst burden in challenged animals.

In conclusion:

1. the parasite can disseminate to cuts of meat intended for the human food chain (chop, loin and forelimb)
2. in pigs that have become infected with the parasite and use of S48 appears to reduce the number of tissue cysts within these tissues.

(Full details of the results of the vaccination trial, including all tissues tested by mouse bioassay and ITS1 PCR can be obtained from the published manuscript (Burrells et al., 2015)).

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2.3. Risk factors in pigs in the UK

2.3.1. Introduction

Pork has been considered to be a major source of *T. gondii* infection in various countries, e.g. in the USA pork and *T. gondii* are ranked second among the top 10 pathogen-food combinations in terms of annual disease burden (Batz et al., 2012).

Frequency of infection in pigs varies between countries and has been associated with housing type (outdoors or indoors), their diets (animals fed on food waste being at higher risk) and contact with cats. Recent trends in consumer habits in developed countries indicate a shift towards consumption of "welfare-friendly" or organic pigs, which increase pigs' exposure to the environment. Pigs in these systems have a higher risk of becoming infected with *T. gondii* than pigs kept in traditional indoor housing (van der Giessen et al., 2007). Therefore, increasing consumption of organic pork may increase the risk of exposure of EU consumers to *T. gondii*.

Both the European Food Safety Authority (EFSA) and the Food Standards Agency's (FSA) Advisory Committee on the Microbiological Safety of Food (ACMSF) have highlighted that more data is needed on the incidence, severity and prevalence of *T. gondii* cysts in carcases and the extent to which meat consumption contributes to human infection.

Aim of the study

The aim of the present study was to identify risk factors for *T. gondii* infection of pigs, in the UK, at the farm level.

2.3.2. Material and Methods

Study design

A cross sectional study was carried out in England between January and July 2015. Farms were recruited through slaughterhouses. Initially, a note explaining the aim and relevance of the study was published in the British Pig Executive (BPEX) newsletter in December 2014. As a result of this, one slaughterhouse with large throughput (more than 2,000 pigs per day) and 4 slaughterhouses with low throughput (less than 100 pigs per day) volunteered to take part of the study. Farms sending pigs to these slaughterhouses were then invited to take part in the study.

Data collection

Blood samples were collected during routine slaughter at the point of bleeding (sticking). Nine millilitres of blood were collected from each pig using pre-labelled vacutainer serum tubes. Up to 25 pigs were sampled per batch/farm. For large batches every third animal was sampled until the required sample of 25 pigs was achieved; while for small batches (less than 25 pigs) all pigs in the batch were sampled. Date of sampling and animal gender were recorded. Blood samples were centrifuged to separate sera from blood cells and sera samples were tested by the Modified agglutination test (MAT) as described in Appendix 6.1.3.

Information on farm characteristics, management practices and biosecurity was gathered using a standardised questionnaire (Appendix D). The questionnaire was either sent by post (with a pre-paid envelope for return) or given to farmers (if the farmer took the pigs to the slaughterhouse himself).

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Farmers had the option to fill the questionnaire on the same day or take it with them to fill out later and return via post (in a pre-paid envelope provided).

Questionnaire data and laboratory results were entered into a relational database in Microsoft Access 2013.

Data analysis
Missing values and errors were checked for during the preliminary descriptive analysis. Descriptive statistics were obtained (i) at animal level for all pigs sampled and (ii) at farm level for variables captured in the questionnaire for farms which completed the questionnaire.

Animal level:
The extent to which gender was associated with animal infection was determined using a multivariable logistic regression including farm as a random effect. Sera titres of 1:25 were considered IgG positive.

Sero-prevalence estimation:
A Bayesian model previously constructed in R (R Core Team, 2015) was used to estimate the probability that each individual farm was positive. Combining the farm results, an estimation of farm prevalence was obtained using the same model (Beauvais et al., in preparation). Briefly, the farm-level prevalence (i.e. the probability of each farm with at least one animal with antibodies against *T. gondii*) was estimated after taking into account the imperfect sensitivity and specificity of the test and the uncertainty arising from sampling a different proportion of animals in each batch. Sera titres of 1:25 were considered positive as a starting point and a sensitivity and specificity of 86% and 95% respectively were considered (Gamble et al., 2005).

Identification of risk factors for *T. gondii* infection at farm level:
Putative predictors of exposure to *T. gondii* within a farm were grouped into categories on the basis of answers given in the questionnaire and risk factors previously identified in the literature (Table 1). Based on the results from the Bayesian model mentioned above, a farm was considered positive if the probability of it having at least one true positive animal was ≥0.50.
The association between predictor variables (Table 14) and farm status was tested by means of Fisher’s exact test; relative risk was calculated as a measure of strength of association.

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

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Table 14: Variables considered as putative risk factors for *T. gondii* infection in commercial pigs. Information collected between January and July 2015 by means of a 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* *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.*	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

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Variable description and question asked in the questionnaire	Categories / options provided in the questionnaire	Variable re-grouped for analysis
	-Individual feeders -Bowl -Pipeline -Other (Please specify):	Off the floor (Yes/No) | the floor and some off floor
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 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?*	-Yes, cats definitely come into direct contact with pigs / pig's food / pigs' drinking water -Yes, it is very likely that cats come into contact with pigs/ pig's food / pigs' drinking water -Not sure	Possible
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
	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

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2.3.3. Results

2.3.3.1. Serological results animal level

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%) but only 75 pigs (3.6%) had titres ≥1:25 (Fig. 2). Gender was not significantly associated with *T. gondii* infection (p=0.14).

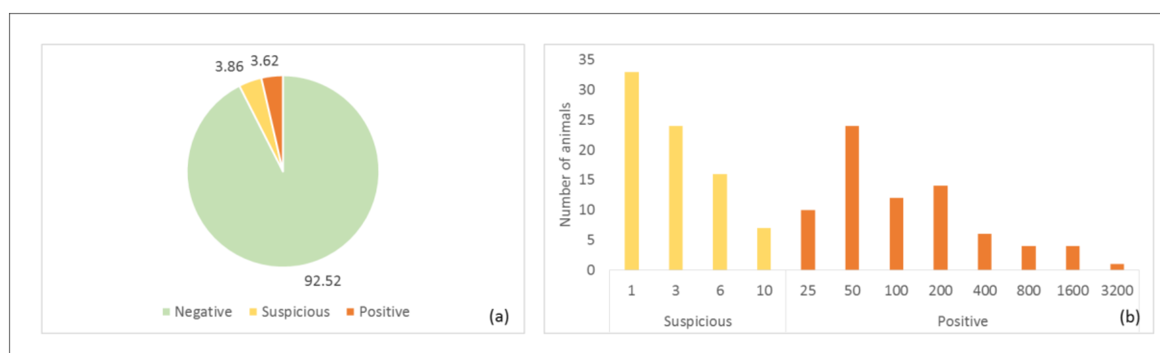

Figure 2: (a)Proportion of negative, suspicious (titre 1:1, 1:3, 1:6 and 1:10) and positive (titre ≥1:25) pigs (n=2017) and (b) number of animals in each titre band. Samples collected between January and July 2015. Results in this figure are not adjusted for the Sensitivity and Specificity of the test.

2.3.3.2. Serological results farm level and characteristics

Twenty-four farms out of 131 sampled had at least 1 animal positive (apparent prevalence 18.3%). The adjusted percentage was 11.5% (95% credibility 8.4%-16.0%) – note that this is a sample estimate not a population estimate.

From those farms that returned a completed questionnaire (n=73), only 5 farms were deemed positive (≥50% probability) (Fig. 3).

Seventy-three farms (55.7%) returned a completed questionnaire. 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 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. Twenty-seven farms (37%) had cats on the site and 62% considered it was possible for cats not belonging to the site getting into the site (Table 15).

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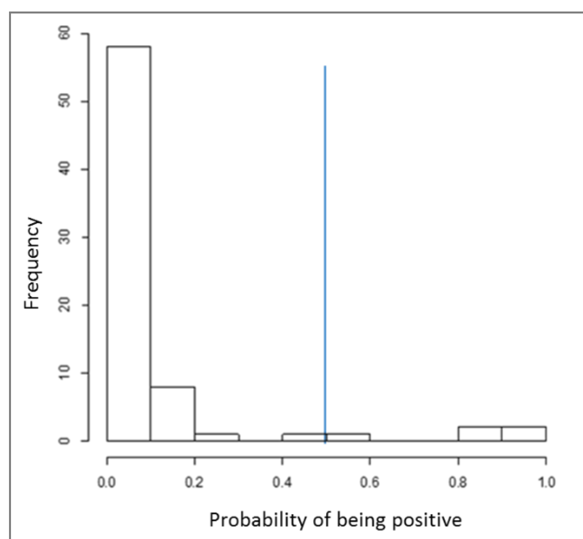

Figure 3: Probability of each farm being positive after adjusting for test sensitivity and specificity and proportion of animals sampled in each batch

2.3.3.3. Risk factors analysis at farm level

There was no statistically significant association (P≤0.05) between positive farms and any of the putative risk or protective factors explored (Table 15). However some patterns did arise, not surprisingly the presence of cats increase the risk 2.5 fold. The relative risk of a farm being positive was higher on those farms having a continuous cycle (RR=2.7), having an open feed storage (RR=5.8), 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. In addition, buying pigs from another farm increase the risk by 4 fold. The power of the study was very low to identify associations as significant given the small number of positive herds. Therefore, although the associations found are not statistically significant, they should be kept in mind.

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Table 15: Distribution of potential risk factors by *T. gondii* positive (n=5) and negative (n=68) farms including results for crude association between *T. gondii* and potential risk factors

Risk factor	No. negative farms	No. positive farms	P value	Relative Risk
Farm characteristics				
Production cycle				
• Complete cycle	45 (95.7%)	2 (4.3%)		
• Part of the cycle	23 (88.5%)	3 (11.5%)	0.34	2.7
Source				
• Same owner	51 (96.2%)	2 (3.7%)		
• Different owner	17 (85.0%)	3 (15.0%)	0.12	4.0
Farm holdings				
• More than 1 site	18 (94.7%)	1 (7.4%)		
• One site	50 (92.6%)	4 (80.0%)	1	1.4
Production system				
• All in all out	26 (89.7%)	3 (10.3%)		
• Continuous	41 (95.3%)	2 (4.7%)	0.39	2.2
Outdoor access (at any production stage)				
• No	36 (94.7%)	2 (5.3%)		
• Yes	32 (91.4%)	3 (8.6%)	0.67	1.6
Farm size				
• Large herds (>200 pigs)	34 (94.4%)	3 (5.6%)		
• Small herds (1-200 pigs)	34 (91.9%)	2 (8.1%)	1	1.2
Hold other livestock species in the farm				
• No	31 (96.9%)	1 (20.0%)		
• Yes	37 (54.4%)	4 (80.0%)	0.38	3.1
Food and water				
Food storage open				
• No	66 (94.3%)	4 (5.7%)		
• Yes	2 (66.7%)	1 (33.3%)	0.19	5.8
Type of feeders				
• On floor (some or all)	31 (96.9%)	1 (3.1%)		
• Off floor only	37 (90.2%)	4 (9.8%)	0.37	3.1
Pigs drinking water is from a stream well or bore				
• No	49 (94.2%)	3 (5.8%)		
• Yes	19 (90.5%)	2 (9.5%)	0.62	1.6
Biosecurity				
Cleaning between batches				
• Yes	28 (90.3%)	3 (9.7%)		
• No	40 (95.2%)	2 (4.8%)	0.65	2.03
Disinfect between batches				
• Yes	29 (90.6%)	3 (9.4%)		
• No	39 (95.1%)	2 (4.9%)	0.65	1.9
Staff designated to work exclusively in certain areas				
• Yes	10 (83.3%)	2 (1.7%)		
• No	58 (95.1%)	3 (4.9%)	0.18	3.4
Keep cats in the farm				
• No	44 (95.7%)	2 (4.3%)		
• Yes	24 (88.9%)	3 (11.1%)	0.35	2.6
Cats not belonging to the farm get into the site				
• No	27 (96.4%)	1 (3.6%)		
• Possible	41 (91.1%)	4 (8.9%)	0.64	2.5
Cats can get in contact with pigs				
• No	29 (93.5%)	2 (6.5%)		
• Possible	39 (92.9%)	3 (7.1%)	1	1.1
Cats can get in contact with pigs' food				
• No	44 (95.7%)	2 (4.3%)		
• Possible	24 (88.9%)	3 (11.1%)	0.35	2.6
Cats can get in contact with pigs' drinking water				
• No	44 (93.6%)	3 (6.7%)		
• Possible	24 (92.3%)	2 (7.7%)	1	1.2
Preventive medicine				
Deworm in at least one production stage				
• No	30 (88.2%)	4 (11.8%)		
• Yes	38 (97.4%)	1 (2.6%)	0.18	0.2

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2.3.4. Discussion and conclusion

A previous study in the UK found 7.4% of pigs tested by serology were positive for anti *T. gondii* IgG using Sabin-Feldman Dye Test (Cheney and Powell, 2013). However the estimate did not account for the test sensitivity and specificity or the number of pigs tested per farm. Our results at the animal level show a lower proportion of infected pigs (3.6%). Most of the positive pigs came from a small number of farms (6.8%).

Pig farms in the UK are heterogeneous with respect to size, production system, outdoor access, and presence of cats. Despite potential differences on the level of exposure to *T. gondii*, the majority of the seropositive pigs had a low MAT titre suggesting low levels of exposure in the farms studied.

The presence of cats and contamination of feed and water are well known risk factors for *T. gondii* infection (Garcia-Bocanegra et al., 2010; Meerburg et al., 2006). Although no significant associations were found in this study, our results are aligned with risk factors reported in previous studies: the relative risk of being positive was higher in those farms having cats or practices that increase the potential of feed contamination with cat's faeces. The relative risk of infection was only 1.6 for those farms with outdoor access; however, the 2 positive farms that kept pigs indoors sourced their pigs from another farm (different owner). Therefore, pigs could have been infected prior arriving at the finishing farm.

Although a formal risk assessment is needed in order to evaluate the potential effects of different control measurs, our results suggest that at preharvest level, strategies targeting a small number of infected farms may be adequate.

The number of farms sampled was limited, which made it difficult to identify risk factors given the low proportion of infected farms. However, this study provides the first approximation of the level of exposure to *T. gondii* in pigs in the UK, and fills some of the data gaps, previously identified by the Food Standard Agency in the ACMSF, regarding level of infection in the UK and assessment of different husbandry practices. The results from this study can be used for sample size calculation in future studies and to populate formal risk assessments.

In summary:

- This study provides the first approximation of the level of exposure to *T. gondii* across different management systems in the UK pig population
- Using MAT titres ≥1:25 as a cut off, the results suggest a low proportion of infected pigs with most infected pigs coming from a small proportion of farms.
- Although no statistical significant associations were found, observed trends were aligned with risk factors previously reported in the literature.

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3. Experimental study in horses in France and Serbia

In horses, a slaughterhouse study was perfomed to determine the concordance between direct and indirect detection of *T. gondii*. This study is described in the following chapter.

3.1. Introduction

Horse meat consumption in EU is less important than other types of meat (lower than 1 kg/capita/year) yet significant in countries such as Belgium, France, Italy or Switzerland (Boireau et al., 2000). It may pose an increased risk due to its raw consumption especially in traditional plates like "*sfilacci di cavallo*" (Italy) or "*tartare*" (France). Eating raw horse meat may expose consumers to *T. gondii*, which may cause life-threatening primary infection or severe congenital toxoplasmosis as has been published in France (Pomares et al., 2011). The aim of this study was to assess the concordance between serology, mouse bioassay and PCR-based detection on tissue digests and magnetic capture qPCR (MC-PCR) in a slaughterhouse study in two countries (France and Serbia).

In horses a low prevalence of tissue cysts is expected (Tassi, 2007). To be able to study the concordance between serological results and presence of tissue cysts, it is necessary to have a reasonable number of bioassay or PCR positives. In general, *T. gondii* prevalence increases with age in animals and humans, however, in horses little is known concerning the isolation of viable parasites. The extensive literature review showed no trend in *T. gondii* isolation in relation to age (young vs old), sex (male vs female) or breed. Therefore a convenience sampling with partial preselection for age was carried out.

To have a reliable estimate of the concordance between serological results and the presence of tissue cysts, one of the samples represented a predilection site. A second sample that is representative of edible tissues was collected to give an indication of the distribution of tissue cysts inmeat. To select the target tissues, a literature review was performed on the anatomical distribution of tissue cysts in horses. The results are presented in Table 1. From this table it is clear that heart and brain are predilection sites for *T. gondii* in horses, and tongue, small intestine, spinal cord and kidneys were shown to be *T. gondii* positive in a limited number of studies. Heart is easier to collect and more suitable for bioassay in mice than brain. Therefore, heart was sampled as a predilection site. Various muscles and meat cuts have been tested and reported but mostly in a limited number of studies. Thus, we took diaphragm as a representative of edible tissue since it represents the only anatomically defined edible muscle tissue among those mentioned in Table 1 (e.g. 'thigh muscles' represents a number of anatomically separate muscles; 'skeletal muscles' provides no precise anatomical description).

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Table 1: *T. gondii* detection in horse tissues, only the tissues that showed positive results are included and ranked by weighted (W) summed score

	Average within study score	Number of studies		Fraction positive studies		Summed score	
		Tissue tested	Positive results	UW[a]	W[b]	UW[a]	W[b]
heart	0.53	3	3	1.00	1.00	1.53	1.53
tongue	0.75	1	1	1.00	0.33	1.75	1.08
small intestine	0.75	1	1	1.00	0.33	1.75	1.08
brain	0.35	3	2	0.67	0.67	1.02	1.02
spinal cord	0.33	2	2	1.00	0.67	1.33	0.99
kidneys	0.23	2	2	1.00	0.67	1.23	0.89
meat/muscle combined	0.23	2	2	1.00	0.67	1.23	0.89
thigh muscle*	0.25	1	1	1.00	0.33	1.25	0.58
skeletal muscle*	0.20	1	1	1.00	0.33	1.20	0.53
diaphragm	0.10	2	1	0.50	0.33	0.60	0.43
lungs	0.08	3	1	0.33	0.33	0.42	0.42
liver	0.07	3	1	0.33	0.33	0.40	0.40

[a]UW: unweighted, [b]W: weighted
* These meat cuts and muscles were included in the “meat/muscle combined” category

3.2. Material and Methods

3.2.1. Sample collection

The sample size was set at 100 horses per country to stay within the sampling and bioassay capacities in each country. To determine the sampling strategy an inventory per country was made showing the existing slaughterhouses and eventually the type, sex and age of animals that are being slaughtered (Table 2).

Table 2: Average number of horses slaughtered in France and Serbia between 2012-2013

	Slaughterhouses	**Age**	**Total**
France	In France there are 91 slaughterhouses that had slaughtered equines in 2012, with 21 of them with a significant mass to be treated (> 50 tonnes), and only two of them (East and South) with more than 500 tonnes.	Horses < 11 years: 7817 (36%) Horses > 11 years: 13646 (64%)	21463
Serbia	In Serbia, several abattoirs for horses exist, but horses are not slaughtered on a daily basis. North of Belgrade, there is a slaughtering house that is slaughtering horses on a weekly basis (one day per week; 2-3 animals). Another one in the vicinity of Belgrade) generally slaughters horses but not on a pre-determined basis.	Horses < 1 year: 20 (5%) Horses > 1 year: 380 (95%)	400

In every country sample collection was performed at slaughterhouse by local staff. In France the sampling was performed at one slaughterhouse (East of France) and in Serbia in two slaughterhouses (North of Belgrade and in the vicinity of Belgrade). Horses were coded with an unique ID and it was ensured that matching samples were labelled correctly. A minimum of 20ml of blood was collected in a 50ml tube at bleeding or from the heart during evisceration. A minimum of 150g of the muscular

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part of the diaphragm and 250g of heart was collected in separate seal bags/plastic containers. Sampling was limited to one animal per farm, and age, sex and breed were noted. Samples were kept and transported on ice or in the refrigerator as much as possible. Hearts were processed for mouse bioassay the day after sample collection in France and Serbia, respectively. Diaphragm samples were stored at -20°C and, later on, were sent to ENVA for MC-PCR testing.

3.2.2. Antibody detection in horses

All horse sera samples collected were send to ANSES – USC EpiToxo in Reims and tested by modified agglutination test (MAT) according to appendix 6.1.3.

3.2.3. Mouse bioassay

Horses were tested by mouse bioassay of the heart in the country of sample collection (one laboratory per country). Trypsin digestion of heart and inoculation in two mice per digest was performed as described in appendices 6.1.1.1 and 6.1.1.2.. At 6 weeks post inoculation, mice were bled from the retroorbital sinus and euthanized. The brain of each mouse was collected aseptically and homogenised for qPCR (France, Serbia) and microscopic examination (Serbia). The mice were tested by MAT for *T. gondii* IgG in each country (ENVA, France and IMR, Serbia). The limit of detection of the microscopic examination is 10 cysts/mL homogenate (i.e. 1 cyst has to be seen in at least one of the 4 homogenate slides) (Djurkovic-Djakovic et al., 2005). DNA was isolated from mouse brain homogenates according to appendix 6.1.2.1 and tested by 529bp RE qPCR according to the same protocol as for PCR on heart digest (see section 1.2.4). 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 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 France and 82bp in Serbia) in gel electrophoresis. A mouse bioassay was considered positive if at least one mouse was positive in PCR or by microscopy or by serology.

3.2.4. PCR on heart digest

DNA was isolated from heart digests using Nucleospin kit as described in appendix 6.1.2.1. Samples were subsequently tested by 529bp RE qPCR. This was performed in the country of sample collection (France and Serbia). 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.

3.2.5. MC-PCR

MC-PCR was performed at ENVA on the frozen edible tissue samples (diaphragms) for all horses (France and Serbia) irrespective of their serological result. MC-PCR was performed as described in appendices 6.1.2.2 and 6.1.2.3. PCR was considered positive using the criteria described for qPCR heart digest (section 3.2.4).

3.2.6. Data analysis

The agreement between the presence of antibodies as determined by MAT and the presence of parasites in horse tissues was evaluated based on kappa-statistics with 95% confidence interval. For this comparison mouse bioassay, PCR on heart digest and MC-PCR on diaphragm were considered separately and as a combined category of overall direct detection positive animals.

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3.3. Results

3.3.1. Collection of horse samples

The proposed sample sizes were reached (and exceeded) in both countries. One-hundred eighty horses were collected in France and one-hundred four in Serbia. The sex and age distribution of the horses is presented by country in Table 3 and by breed/type in Table 4. Young is considered as <2 years, and adult ≥ 2 years of age. As expected the proportion of male and female horses were similar amoung the slaughtered adult horses. In Serbia, most slaughterd horses were adults. The main breed/type that was collected was trait horse in both age-categories.

Table 3: Mean age and sex of horses sampled in Serbia and France

			Young Count	Mean age (years)	**Adults** Count	Mean age (years)
Country	France	NN*	0	0	1	3
		Female	17	2	60	7.9
		Male	37	1	65	5.7
		Total	54	1	126	6.7
	Serbia	Female	3	1.3	48	6.0
		Male	1	1	52	6.5
		Total	4	1.2	100	6.2
Total		NN*	0	0	1	3
		Female	20	1.5	108	7.1
		Male	38	1.4	117	6.04
		Total	58	1.4	226	6.5

* Exact age and sex were missing for one adult (based on slaughterhouse) from France

Table 4: Breed/type of horses sampled in Serbia and France

Breed/type	**France**		**Serbia**		**Total**
	Young	**Adult**	**Young**	**Adult**	
Anglo arabe	1	0	0	0	1
Arabe	1	3	0	0	4
Auxois	0	1	0	0	1
Breton	0	1	0	0	1
Cob	9	1	0	0	10
Grade	0	0	4	74	78
Lipizzaner	0	0	0	1	1
Percheron	2	1	0	0	3
Pony	2	0	0	0	2
Purebred	0	12	0	0	12
Saddle horse	6	28	0	0	34
Trait	21	79	0	0	100
Working	0	0	0	7	7
Working, heavy	0	0	1	17	18
Not identified	7	5	0	0	12
	49	**131**	**5**	**99**	**284**

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3.3.2. Detection of antibodies against *T. gondii* in horses

Antibodies (IgG) were detected by MAT in 35.5% of horses using a cut-off value ≥1:6 (Table 5). Higher prevalence was observed in animals from Serbia (48.1%) compared to France (27.8%) showing statistical significance between origin of the horses and serology results (Pearson's χ^2 *p*-value=0.001). Titers were low, and the maximum titer was 1:200 or above 200 for 4 adult horses (3 years old, France and 5, 8, and 9 years old, Serbia) and one young (12 months, France). No statistical difference in seroprevalence was observed between young and old horses (Pearson's χ^2 *p*-value=0.195) (Table 6).

Table 5: Modified agglutination test (MAT) titers and classification (cut-off value ≥1:6) for horses from France and Serbia. (*Two sera were absent from MAT analysis)

MAT	France	Serbia	Total
negative	128	54	182
positive	50 (27.8%)	50 (48.1%)	100 (35.5%)
1:6	20	31	51
1:10	11	7	18
1:25	12	6	18
1:50	2	1	3
1:100	3	2	5
1:200	0	1	1
>1:200	2	2	4
	178	104	282*

Table 6: Modified agglutination test (MAT) titers and classification (cut-off value ≥1:6) for horses by age (*Two sera were absent from MAT analysis)

MAT	Adult (≥ 2 years)	Young (< 2 years)	Total
negative	143	39	182
positive	85 (37.3%)	15 (27.8%)	100 (35.5%)
1:6	42	9	51
1:10	16	2	18
1:25	16	2	18
1:50	3	0	3
1:100	4	1	5
1:200	1	0	1
>1:200	3	1	4
	228	54	282*

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3.3.3. Direct detection of *T. gondii* in horses

The presence of *T. gondii* was demonstrated in the tissues of horses in both countries (Table 7). In France, 2 horses were reported as positive in qPCR on mouse brain, 1 by qPCR on heart digest and 13 by MC-PCR on diaphragm. All horses with positive diaphragms had negative results inn qPCR on heart digest or qPCR on mouse brain. Only one horse (CH102) presented an overlapping result a positive result in qPCR on heart digest and in qPCR on mouse brain (only 1 out of 2 inoculated mice).

In Serbia, 48 horses were reported as positive in qPCR on mouse brain, 10 in microscopy on mouse brain, 28 on heart digest and 8 on diaphragm. Several overlapping results were obtained: 16 samples had a positive result both in qPCR on heart digest and mouse bioassay; 2 samples in mouse bioassay and MC-PCR on diaphragm; 1 sample in MC-PCR and qPCR on heart digest. Only 2 horses (N° 1 and N°4) presented a positive result for mouse bioassay, qPCR on heart digest and MC-PCR. Both were negative in MAT. Two *T. gondii* strains have been isolated by mouse bioassay in Serbia and the description of the respective horses is shown in Table 8.

Table 7: Detection of *T. gondii* in the tissues of horses from France and Serbia

	France	Serbia	total
Mouse bioassay*	2/175	56/103	58/278
PCR mouse brain	2/175	48/103	50/278
Microscopy on mouse brain	ND	10/103	10/103
Serology mice	0/175	2/103	2/278
PCR heart digest#	1/175	28/104	29/279
MC-PCR diaphragm	13/180	8/104	21/284
Any direct detection method	15/180	69/104	84/284

*Mouse bioassay: In France, 5 bioassays have not been accomplished. In Serbia, for one bioassay, both mice died 2-3 dpi, with a large number of bacteria isolated; one mouse corresponding to another bioassay died at 17 dpi and was not further investigation. # In France, 5 heart digest PCRs have not been performed.

3.3.3.1. Results mouse bioassay

a. Microscopy: the technique has been performed in 103 samples, and only in Serbia. In 10 out of 103 (9.7%) samples, tissue cysts were observed. Mice from two bioassays presented abundant cysts (320-1010 cysts/ml), while mice from one bioassay each had 30, 40 and 50 cyst/ml, and mice from four bioassays had 10 cysts/ml each. Another 6 samples presented inconclusive results with tissue cyst-like structures, which were discarded from the final analysis.

b. PCR on mouse brain: In France, the presence of *T. gondii* was demonstrated for two bioassays. In Serbia, the PCR analysis on mouse brains suggested 48 positive bioassays. Due to the large discrepancy in the proportion of positive findings between France and Serbia, a ring trial was organised by RIVM as the coordinator on qPCR among these 2 countries. Based on the results of the ring trial, it was decided to test all Serbian mice brain samples in France. PCR on brain samples, performed in France, was positive for 48 Serbian horses. PCR contamination in France could be excluded because of the use of different PCR primers and the amplification of a larger PCR fragment. However due to the discrepancy with the bioassay and MC-PCR results in both countries, it was concluded not to use these results for the final analysis

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c. Serology on mice sera: All mice gave negative results by MAT analysis of sera, both in France and Serbia, except for the mice corresponding to the isolated strains (n=2) from Serbia, that presented high titers (5120 and 20480).

3.3.3.2. qPCR on heart digests

In France the presence of *T. gondii* was demonstrated in a tissue sample of one horse. In Serbia PCR positive results on heart digests were obtained in 28 samples. Due to these very wide differences, the original heart digest samples from Serbia were re-tested in France, and the same number of positive results (28) was obtained. These results were also not used for the final analysis (see above).

3.3.3.3. MC-PCR of diaphragm

All MC-PCR on horse diaphragm were performed in France. Magnetic capture of the 529bp RE of *T. gondii* was performed on the diaphragms from 284 horse samples. The presence of *T. gondii* was demonstrated in the tissues of 21 horses: 13 from France and 8 from Serbia. Eight samples (both countries) were originating from seropositive animals, while 12 samples (both countries) were coming from seronegative animals and for one horse the MAT analysis was missing.

Due to the discrepancy with the direct detection using PCR on mice brains and qPCR on heart digests in Serbia, it was decided to use only microscopy (10/103) and MC-PCR results for further analyses.

Table 8: Characteristics of horses with a positive strain isolation of *T. gondii*

Animal ID	Country	Age	Sex	Type	Direct detection	Indirect detection
RS39	Serbia	6 years	F	Grade	• 2 mice PCR+ • 2 mice microscopic examination+ • heart digest PCR+ • MC-PCR -	Neg
RS40	Serbia	4 years	F	Grade	• 2 mice PCR+ • 1 mouse microscopic examination+ • heart digest PCR- • MC-PCR on horse diaphragm -	Pos (1/6)

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

The direct detection results are presented by serological status in Table 9 to 11. A MAT titer ≥1:6 was considered positive. There is a lack of concordance between presence of IgG and detection of *T. gondii* using (any) direct detection methods. For the concordance between MAT and mouse bioassay the Cohen's kappa-value is estimated at 0.08 (95%CI: -0.08-0.23), for MAT and qPCR on heart digest at 0.07 (95%CI: -0.07-0.21) and for MAT and MC-PCR at 0.02 (95%CI: -0.13 – 0.17). The overall direct detection rate in seropositives (16.3%) is 2 times higher than the detection rate in seronegatives (8.7%), however this difference is statistically not significant (Pearson's χ^2, *p*-value = 0.08).

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Table 9: Concordance between detection of IgG by MAT in horses and demonstration of viable *T. gondii* in heart by mouse bioassay

	Bioassay		
	positive/total	positive/total	
MAT	Serbia*	France**	total
negative	2/54	2/124	4/178
positive	8/49	0/49	8/98
	10/103	2/175***	12/276
Cohen's kappa (95%CI)	0.13 (-0.07 – 0.33)	-0.02 (-0.26– 0.21)	0.08 (-0.07 - 0.23)

*Only microscopic examination on mouse brain was taken into account for this analysis
** Only results of qPCR on mouse brain were taken into account for this analysis
*** Two MAT results were missing

Table 10: Concordance between detection of antibodies by MAT in horses and detection of *T. gondii* DNA in 100g of diaphragm by magnetic capture and qPCR

	MC-PCR		
	positive/total	positive/total	
MAT	Serbia	France*	total
negative	7/54	5/128	12/182
positive	1/50	7/50	8/100
	8/104	12/178	20/282
Cohen's kappa (95%CI)	-0.11 (-0.31 – 0.08)	0.13 (-0.08 – 0.34)	0.02 (-0.13 – 0.17)

Table 11: Concordance between detection of antibodies by MAT (IgG) in horses and detection of *T.gondii* by any direct detection method (qPCR on mouse brain-FR; microscopy on mouse brain-RS; qPCR on heart digest-FR and MC-PCR on diaphragm-FR+RS)

	Any direct detection method	**Overall detection rate**
	positive/total	positive/total
MAT	total	total
negative	16/184	8.7%
positive	16/ 98	16.3%
	32/282	11.35%
Cohen's kappa (95%CI)	0.09 (-0.05 – 0.23)	

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3.4. Discussion and conclusion

Serum, heart and diaphragm samples have been collected from horses slaughtered in two countries. In both countries the targeted sample size of 100 horses per country has been reached. In France, initially horses were collected by convenience and after sampling the first 100 horses, we collected 41 young (< 2 years) and 59 adults (≥ 2 years). Compared to the ratio of the slaughtered animals in the previous year (2014) of 25.5% young and 74.5% adult horses, an age-disproportion (%) was revealed. Therefore we went up to a final collection of 180 horses, adjusting though the percentages of young/adult to 31.1: 68.9, closer to the ratio of the previous year. As expected, slaughtered adults were equally male and females, whereas slaughtered young horses were mainly male, since the young females (<2 years) were kept for breeding. The breed/type that was predominant was "trait" horse (work horse, heavy), most likely due to usually large muscular mass in this type of horses. However the collection of samples was performed in only one slaughterhouse (out of 91 nationwide) in the East of France, during a short period of time (10 weeks) and thus is not representative for the French slaughtered horse population in general. In Serbia most of the animals were adults (100), which were collected at a rate of 3-4 animals/week in 2 different abattoirs. It is expected that sampling in Serbia was most likely representative for the slaughtered horse population of Serbia.

The aim of the study was to analyse the concordance of antibody detection and presence of (viable) parasites. We had chosen the very lowest dilution (1:6), as a positive cut-off for sera. Since MAT is a species-independent serological test that is commonly used with animal matrices to detect specific *T.gondii* IgG antibody presence, we considered the very first dilution (1:6) as a trustworthy indicator of such a specific reaction. An overall seroprevalence of 35.5% was obtained based on MAT on sera and the seroprevalence in France (27.8%) was significantly lower than in Serbia (48.1%) (Pearsons χ^2 p-value=0.001). It should be kept in mind that this might be biased by the fact that horse samples were collected in France in only one abbatoir (out of 91 nationwide), and consequently might be not representative of the whole horse population of France.

Antibody titers were low, similarly to cattle, with the maximum titer of 1:200 or above 200 for 5 animals only. Seroprevalence was lower in young horses (27.8%) compared to adult horses (37.3%). Various direct detection methods were used to demonstrate the presence of *T.gondii* in the tissues of horses in both countries: mouse bioassay from heart digest (qPCR on mouse brain andmicroscopic examination on brain's homogenate in Serbia), qPCR on heart digest and MC-PCR on diaphragm. Except for MC-PCR that was performed only in France, the rest of the methods have been performed in each country (microscopic examination only in Serbia). Overall, in France, 15 horses (8.15%) were reported as positive in at least one of the direct techniques, while they were 69 positive horses in Serbia (66.34%). Although it might reflect a true difference between the two countries as the number of seropositives was two times higher in Serbia, we think that these differences might be better explained by two other hypotheses because the number of direct detection positives by qPCR in France and Serbia are very different. : Either a more sensitive method was used by the Serbian laboratory and lack of sensitivity in the French laboratory or a contamination issue. A ring trial had been performed by sharing several negative and positive samples from France and Serbia, between the coordinator (RIVM, NL) and the two laboratories. When analyzing the results of the ring trial, together with the fact that all partners used standardized PCR protocols, the contamination issue could not be excluded. As it was unclear at what point in the process contamination occurred and contamination during sample preparation could not be ruled out, we preferred to take into the final analysis only the 10 mouse bioassay positives for 103 analyzed by microscopy for Serbia and 8 MC-PCR positives for 104 horses analysed. The technique of microscopic examination when compared to the qPCR on mouse brain, is assumed to be less sensitive, therefore the number of horses scored positive by mouse bioassay from Serbia may be an underestimation. However when we look at the

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MC-PCR detection rate, performed in only one laboratory, on one matrix from 2 different geographical origins, we found similar results: 7.1% for France and 7.7% for Serbia.

Overall, 33 animals were considered direct detection-positive (11.6%), and for 12 of them this conclusion is based on mouse bioassay (4.2%), indicating the presence of viable tissue cysts. All mice gave negative results when analysed by MAT, except for the mice corresponding to the isolated *T. gondii* strains from Serbia, that presented high titers (5120 and 20480), suggesting though that even when present, the parasite concentration is generally low in horse heart. As a consequence, low inoculation doses for the mice have resulted in low grade infections in the mice, low parasite loads in the mouse brain homogenates and failure to elicit a detectable antibody response in mice.
The results for 21 horses are based on PCR methods performed on either heart digests, mouse brain homogenates or diaphragms. To exclude potential contamination, appropriate blank controls were included during DNA isolations and PCR runs, and always confirmed negative. Non-specific amplification was excluded by considering only samples with an amplification curve similar to the positive controls and a Cq-value below 40. For samples that became positive between cycle 35 and 40, the amplicon size was checked by gel electrophoresis. Due to the low DNA concentrations in the amplified samples, genotyping PCRs were not feasible.
Overall there was a lack of concordance between direct and indirect detection methods (0.09) and more detailed using MAT and bioassay (0.08) and MAT and MC-PCR (0.02). This result in horses is comparable with the results as reported for cattle in the literature review and in the slaughterhouse study in cattle in four countries (chapter 2). The modified agglutination test was selected for the detection of antibodies (IgG) in horses. This is a species-independent serological test that is commonly used with various types of animal sera and other tissue fluids in horses. In France, horse sera had additionally been tested using ID.Vet ELISA test, the results of which do not correlate well with MAT, but also do not correlate with the direct detection results (data not shown).

In addition to a lack of concordance between direct and indirect detection of *T. gondii*, also the results from the different direct detection methods showed discrepancies. Diaphragm samples were tested by MC-PCR, whereas heart digests were tested by qPCR and inoculation in mice. Therefore, the lack of concordance between these methods may indicate that horses are not necessarily equally infected with *T. gondii* in both tissues and it suggests that the overall parasite load is low. This lack of concordance might have to do also with the relation between parasite kinetics and sample size: in pigs or ovines, the heart is at least 3 times smaller than in horses and, if assumed that the number of parasites is the same, this would result in a lower final concentration.

The heart was chosen as predilection site and diaphragm as representative of edible tissue. From the results presented here, it can be concluded that horses are unlikely to be infected to a detectable level in both tissues simultaneously. However, because different methods were used for the two tissues it is not possible to conclude that one tissue is more likely to be infected than the other.

Conclusions

1. Of the horses, 11.6% were considered positive for *T. gondii* by direct detection, and for 4.2% of them this conclusion is based on mouse bioassay, indicating the presence of viable tissue cysts.

2. Overall, there was a lack of concordance between direct and indirect detection methods. In horses, the antibody detection by MAT and the direct detection of *T. gondii* were not correlated irrespective of the direct detection methods applied in horses.

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3. There is no concordance between different direct detection methods, most likely reflecting a low concentration of *T. gondii* in positive horses in combination with the different organs used for mouse bioassay and MC-PCR.

4. There was a significant difference between the qPCR based direct detection on mice brains and heart digests results in France (0.6%) and Serbia (46.7%), but not on MC-PCR of diaphragms. Therefore, it was concluded that the unexpected high number of bioassay positives in Serbia could be due to a technical problem and therefore only the microscopical results were included in the final analyses. Since there was a significant difference in seroprevalence, further studies are needed in Serbia to assess the potential risk.

5. Two isolates of *T. gondii* has been achieved from horses in Serbia, which demonstrates the presence of viable *T. gondii* in horses.

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4. Experimental studies in poultry

Two studies were performed in poultry and are described in this chapter. First, similarly to the cattle and horse studies, the correlation between indirect and direct detection of *T. gondii* was studied in naturally infected animals (section 4.1). In contrast to the cattle and horse studies, chickens were obtained directly from organic and backyard farms, instead of through the slaughterhouse. The farmers completed a questionnaire for risk factor analysis. In the second study, the anatomical distribution of *T. gondii* in experimentally infected chickens and turkeys is determined by MC-PCR testing of various tissues (section 4.2).

4.1. Slaughterhouse study in organic laying hens

4.1.1. Introduction

Chickens and turkey are known to be highly susceptible to *T. gondii* infection, however, industrialized husbandry has decreased *T. gondii* exposure for poultry. With the increasing demand for organic meat, the risk of human infection from poultry meat is potentially on the increase. Furthermore, there is an increasing market share of chicken meat preparations such as chicken burgers and sausages, increasing the probability of consuming undercooked products.

Aim of the study

The concordance between serology and detection of tissue cysts was evaluated in a field study focusing on laying hens from organic farms. The study on laying hens in Germany was performed to identify *T. gondii* positives and to investigate the serum of these animals with various serological tools and the tissues of these animals via PCR for the presence of *T. gondii* or via mouse bioassay for the presence of infective *T. gondii* tissue cysts. The aim was not to investigate prevalence of *T. gondii* infections in hens but to establish to which extent seropositivity was linked to the presence of infective tissue cysts in selected tissues.

In addition, a questionnaire was used to characterize the farms and to obtain information on potential risk factors to explain the serological prevalence of *T. gondii* on the farms.

Two types of tissues were collected. We sampled a predilection site, as this gave us the most reliable estimate on the concordance between serological results and the presence of tissue cysts in meat. The second sampled tissue was representative of edible tissues. This gave us an indication on the distribution of tissue cysts in meat.

The anatomical distribution of tissue cysts had been studied as part of our extensive literature review. From these results it was clear that heart is a predilection site for *T. gondii* in chicken. Drumsticks (lower leg limb muscle) represented the edible tissue with the highest score. The lower leglimb musculature was used as a representative of edible tissues.

To determine the presence of *T. gondii* tissue cysts a mouse bioassay was applied to both, heart and drumstick musculature. The presence of *T. gondii* was further analysed by a Magnetic Capture-PCR (MC-PCR, i.e. a quantitative PCR on heart and drumstick) and by conventional PCR (on the remnants of the pepsin digest which had been used for bioassay).

Three serological methods were applied to analyse hens for antibodies against *T. gondii*, MAT, IFAT and ELISA. Tests, like MAT and IFAT are very often used to examine animal sera and these previous data suggest at least acceptable sensitivity and specificity for antibody detection. As an ELISA we

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chose an ELISA based on TgSAG1, a major surface antigen of *T. gondii* tachyzoites. It is a major target of antibody response during acute but also during chronic infection when again tachyzoites are present because of reactivation events. The TgSAG1 ELISA had successfully applied by FLI to examine ducks and geese for anti-*T. gondii* IgG antibodies.

In addition to serum also the presence of antibodies in fluids of musculature of hens was analysed for the presence of antibodies against *T. gondii* and whether these fluids could be useful to correctly identify laying hens positive either by mouse bioassay or by PCR. In addition to heart and drumstick also breast musculature was sampled, because from this large part of muscle fluids can be collected easily.

4.1.2. Material and Methods

Farms: All sampled laying hens derived from organic farms because we expected a low prevalence of *T. gondii* infection in conventional farms. In addition to large scale farms with up to 3,000 hens per unit also small scale farms (i.e. backyard farms) were sampled. All farms exclusively reared chicken. Farms were selected by convenience because the main aim of the study was to determine the extent of concordance between serology and detection of tissue cysts.

Animals and sampling of animals: Samples were collected from hens slaughtered at the end of the laying period. The ~1 up to 3 year old hens had either been purchased alive on farm or their carcasses immediately after having been slaughtered. Hens were not randomly selected but by convenience. In case of live chicken, these were transported to the animal facilities of the Institute of Parasitology, Faculty of Veterinary Medicine, University Leipzig. After arrival the birds were blood sampled and blood or serum samples immediately shipped over-night to Friedrich-Loeffler-Institute (FLI). At Friedrich-Loeffler-Institute serum samples were immediately analysed by ELISA for the presence of specific antibodies against *T. gondii*. It was planned to examine 30 positive and 30 negative hens by bioassay, by PCR and further serological assays. Thus, out of the hens analysed by ELISA the required numbers of hens with a positive result as well as hens with a negative serological result were selected for further analysis.

Information from the extensive literature review and the experimental studies were used to select optimal tissues to be analysed by MC-PCR, bioassay and meat juice testing. Heart, the drumsticks (lower leg limb musculature) and the breast musculature were collected and equal portions of each organ were prepared and shipped cooled but not frozen within 1 or up to 3 days to the participating laboratories (FLI or University Leipzig). The portions for MC-PCR analysis were stored frozen at -20°C until used. Meat juice was either collected after the breast musculature had been chopped and stored cool over night or after stored frozen.

Sample size: The study aimed to identify at least 30 ELISA positive and another 30 control chickens. Based on the assumption that the overall prevalence of *T. gondii* positive hens is 10% the minimum sample size was 400 hens. Up to 6 ELISA positive hens were selected per farm. In addition, also up to 10 chickens per farm with a negative ELISA result were randomly selected for control.

Mouse bioassay: Because the trypsin-based protocol used by other groups of the consortium for tissues of other animal species resulted in chicken tissues in a highly viscous suspension it was decided to use a pepsin-based protocol which had been successfully applied by others and the own working group previously. In contrast to other groups of the consortium, genetically *T. gondii* susceptible gamma-interferon-knockout mice (GKO mice) or gamma-interferon-receptor-knockout mice (GRKO mice) were employed for the project. GKO mice have been successfully used previously for such studies; due to an unexpected problem in breeding GKO mice, in a part of the mouse bioassay GRKO mice

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were used in parallel to GKO mice. There was no statistically significant difference in the proportions of positive bioassays in GKO and GRKO mice, as shown later in detail. Heart and drumstick muscle were pepsin-digested and for each tissue two mice were inoculated, i.e. four mice were inoculated per hen. See Appendix 6.5.1 Poultry1.

Magnetic capture PCR: Magnetic capture PCR was partially performed with equipment different from the equipment described in the original literature. Consequently the protocol had to be adapted. See Appendix 6.5.2 Poultry2.

DNA extraction from pepsin-digested tissues and mouse brains: DNA extraction was performed as described in the Appendix 6.1.2.1.

Conventional real-time PCR: DNA extracted from the remnants of pepsin-digested tissues were analysed by a previously published real-time PCR (Talabani et al., 2009).

TgSAG1-ELISA for chicken and mice: Please find the protocol in the Appendix Poultry3.

IFAT: Please find the protocol in the Appendix Poultry4.

Modified agglutination test (MAT): MAT was performed as described in the Appendices. The positive threshold titre in serum analysis was 1:6. For the analysis of body fluids of chicken examined in bioassay and MC-PCR a titre of 1:1 was selected as positive threshold titre.

Kappa statistics and Youdens's index (YI) were used to characterize the diagnostic potential of the different assays. Kappa with linear weighting were calculated using an online tool (http://www.vassarstats.net/kappa.html). YI was calculated based on sensitivity (Se) and specificity (Sp) relative to reference standard (YI=Se+Sp-1).

Questionnaire: A questionnaire was performed on each of the farms at the day of sampling or purchase of hens. A translation of the questionnaire is provided in the appendices (Appendix Poultry5). Farms were classified into large and small based on the number of chicken reared. Farms with more than 500 laying hens were regarded as large while those with less than 50 laying hens were regarded as small, farm sizes in-between did not occur. Three age-categories were arbitrarily defined: ≤ 12 months (animals were not younger than 10 months), 12-24 months and >24 months.

4.1.3. Results

4.1.3.1. Serological and PCR analyses

Results in TgSAG1-ELISA, IFAT, MAT using chicken serum

A total number of 470 laying hens had been analysed serologically in this study. Due to lack of serum in some of the hens not all of them could be analysed in the remaining serological tests (IFAT, MAT).

The highest apparent seroprevalence was determined by IFAT and the lowest by MAT. Marked differences in prevalences in all tests were observed between large and small holdings. Hens sampled from small farms were more often positive compared to hens sampled from large farms (Table 1, 2, 3). In all serological tests the highest seroprevalences were observed in age category 3 (> 24 months old). In all tests low prevalences were observed in the age categories 1 (≤ 12 months old) and 2 (> 12-24 months old). Since all animals from age category 3 derived from small farms, age dependency was analysed per farm type. In the ELISA young animals (category 1) from large farms showed a higher seroprevalence compared to elder animals (category 2). In small farms, the opposite

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was the case. This effect was not observed in IFAT and MAT. In small farms all tests revealed the lowest prevalence in hens of age category 1 and the highest seroprevalence in hens of age category 3 (Table 1, 2, 3).

Table 1: *T. gondii*-MAT results stratified according to the age of hens and farm type

Farm type		**MAT result**		
Large farm	**Age category**	**POSITIVE (≥6)**	**Neg. (<6)**	**Total**
	1	0 (0%)	35 (100%)	35 (100%)
	2	9 (3%)	324 (97%)	333 (100%)
	Subtotal	9 (2%)	359 (98%)	368 (100%)
Small farm	1	0 (0%)	22 (100%)	22 (100%)
	2	6 (38%)	10 (63%)	16 (100%)
	3	10 (50%)	10 (50%)	20 (100%)
	ND*	13 (57%)	10 (43%)	23 (100%)
	Subtotal	29 (36%)	52 (64%)	81 (100%)
	Total	38 (8%)	411 (92%)	449 (100%)

*No data

Table 2: *T. gondii*-IFAT results stratified according to the age of chicken and farm type

Farm type		**IFAT result**		
Large farm	**Age category**	**POSITIVE (≤50)**	**Neg. (<50)**	**Total**
	1	2 (6%)	33 (94%)	35 (100%)
	2	26 (7%)	321 (93%)	345 (100%)
	Subtotal	28 (7%)	354 (93%)	380 (100%)
Small farm	1	6 (27%)	16 (73%)	22 (100%)
	2	13 (72%)	5 (28%)	18 (100%)
	3	17 (85%)	3 (15%)	20 (100%)
	ND*	20 (77%)	6 (23%)	26 (100%)
	Subtotal	56 (65%)	30 (35%)	86 (100%)
	Total	84 (18%)	384 (82%)	466 (100%)

*No data

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Table 3: *T. gondii*-ELISA results stratified according to the age of hens and farm type

Farm type		**ELISA result (TgSAG1)**		
Large farm	**Age category**	**POSITIVE (> 0.2)**	**Neg. (≤ 0.2)**	**Total**
	1	7 (20%)	28 (80%)	35 (100%)
	2	15 (4%)	334 (81%)	349 (100%)
	Subtotal	22 (6%)	362 (94%)	384 (100%)
Small farm	1	1 (5%)	21 (95%)	22 (100%)
	2	10 (56%)	8 (44%)	18 (100%)
	3	15 (75%)	5 (25%)	20 (100%)
	ND	21 (81%)	5 (19%)	26 (100%)
	Subtotal	47 (55%)	39 (45%)	86 (100%)
	Total	69 (15%)	401 (85%)	470 (100%)

*No data

Agreement between serological test results

The agreement was characterized using un-weighted Kappa. The comparison of MAT and ELISA was characterized by a Kappa of 0.51 (0.37-0.64, 95% CI, moderate agreement). The comparison of MAT and IFAT resulted in a Kappa of 0.50 (0.37-0.62, 95% CI; moderate agreement). The comparison of ELISA and IFAT results revealed a Kappa value of 0.51 (0.40-0.62, 95% CI; moderate agreement).

Results of bioassay

A total number of 61 hens had been selected for bioassay and MC-PCR. Selection was based on the ELISA results because with ELISA we were able to screen and select hens very fast for the bioassay.

In case of heart muscle a mean of 3.02 g of musculature were lysed which resulted in a final mean volume of 2.72 ml of digest of which 0.5 ml per mouse were used for “bioassay”. Mice were followed for 42 days and then necropsied. Once a mouse developed signs of toxoplasmosis (ruffeled hair, apathy) it was euthanized and necropsied. Brain was examined by PCR to detect *T. gondii* DNA and serum analysed by ELISA to find antibodies against *T. gondii.* Peritoneal fluid (10 µl) and a lung homogenate (10 µl) were analysesd light microscopically for the presence of tachyzoites. All mice regarded as bioassay positive were light microscopically positive for *T. gondii* and showed either a positive PCR or a positive serological result. In case of drumstick a mean of 35.2 g of musculature were lysed which resulted in a final mean volume of 31.6 ml of digest of which 0.5 ml per mouse were used for “bioassay”.

Thirty-two ELISA positive hens were selected for mouse bioassay and MC-PCR and 29 ELISA negative (n=20) or inconclusive (n=9) chicken were also analyzed by mouse-bioassay and MC-PCR.

For 26 of these hens the bioassay revealed a positive result. From each hen the pepsin-digest of heart musculature and drumstick musculature was inoculated into two mice each (IFN gamma -/- (C.129S7(B6)-Ifng$^{tm1Ts/J}$)) [GKO] or IFN gamma receptor −/− mice (B6.129Sv/Ev-Ifngr^{tmAgt})

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[GRKO]). Between 15 and 37 days after inoculation the animals developed toxoplasmosis and were killed humanely. Light microscopical analysis of peritoneal washings and lung homogenates revealed tachyzoites in each of the mice. Peritoneal washings and lung homogenates were inoculated on cell culture and each of the cultures became positive. All mice which developed toxoplasmosis were serologically positive in a TgSAG1 ELISA (positive cut-off: 0.1) while all other mice which survived until the end of the observation period (42 days) remained serologically negative in this ELISA. At the end of the observation period all surviving mice were necropsied, their brains completely digested and an aliquot DNA extracted. None of the brains of surviving mice revealed a positive realtime PCR positive result (Cq < 35.0). Eleven surviving mice showed reaction Cq ≥ 35 but when amplification products were examined in 1.5% agarose gel analysis bands did not show the expected size; the PCR results on these mice were thus recorded as negative.

For 23/26 hens only the heart musculature became bioassay positive. In 7/24 inoculations of heart digest into two mice only one of the mice developed toxoplasmosis. In case of 3/26 bioassays, in addition to the inoculation of heart also drumstick revealed a positive bioassay (Table 7). In two of these cases both mice inoculated with drumstick developed toxoplasmosis while in one case only one of two mice developed disease.

In one bioassay, a mouse died unexpectedly 28 days post inoculation. In another bioassay, a mouse died unexpectedly 15 days post inoculation. In both cases cell culture and PCR on tissues of these mice revealed a negative *T. gondii* result.

In 160 of a total number of 244 bioassay inoculations (two tissues, i.e. drumstick and heart per hen; two mice per tissue; consequently four mice per hen), only GKO mice were used. In the remaining 84 bioassay inoculations, both GKO and GRKO were used in parallel (42 each). Nine isolates were achieved by using GKO and six isolates by using GRKO. The differences between these two mouse strains regarding the proportion of positive bioassays were statistically not significant.

Agreement between serologic results and bioassay (Table 4)

ELISA: Thirty-two ELISA positive hens were selected for mouse bioassay and MC-PCR and 29 ELISA negative hens were analyzed by mouse-bioassay and MC-PCR. 71.9% of the ELISA-positive chickens rendered a positive bioassay. Sensitivity of ELISA was 88.5% (23/26) and specificity of ELISA was 74.3% (26/35). The agreement between bioassay and ELISA was characterized by a Kappa of 0.61 (0.41-0.81, 95% CI) (substantial concordance).

MAT: Twenty-one MAT positive hens had been selected for mouse bioassay and MC-PCR. In addition, 40 MAT negative chickens were also analyzed by mouse-bioassay and MC-PCR. 81.0% (17/21) of the MAT-positive chickens rendered a positive bioassay. However, also 22.5% (9/40) of the MAT-negative chickens showed a positive bioassay. Sensitivity of MAT was 65.4% (17/26) and specificity of MAT was 88.6% (31/35). The agreement between bioassay and MAT was characterized by a Kappa of 0.55 (0.34-0.77, 95% CI) (moderate concordance).

IFAT: Thirty-three IFAT positive hens had been selected for mouse bioassay and MC-PCR. Further 28 IFAT negative hens were also analyzed by mouse-bioassay. 72.7% (24/33) of the IFAT-positive chickens rendered a positive bioassay. Only 7.1 % (2/28) of the IFAT-negative chickens showed a positive bioassay. Sensitivity of IFAT was 92.3% (24/26) and specificity of IFAT was 74.3% (26/35). The agreement between bioassay and IFAT was characterized by a Kappa of 0.64 (0.45-0.83, 95% CI) (substantial concordance).

Majority of test results: If the classification is done based on the majority of serological test results, 28 positive hens had been selected for bioassay and MC-PCR. 85.7% (24/28) of the majority-positive

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hens revealed a positive bioassay. In addition, 33 majority-negative chickens were selected; 6% (2/33) of these chickens revealed a positive bioassay. Sensitivity of the reference MajRef was 92% (24/26) and specificity of this reference was 89% (31/35). The agreement between bioassay and MajRef was characterized by a Kappa of (0.80, 0.65-0.95, 95% CI) (substantial concordance).

Table 4: Summary on the comparison between the results in serological tests and bioassay

Test (analyte)	Kappa	Kappa (95% CI)	Sensitivity	Specificity	Youden's index
MAT (serum)	0.55	0.34-0.77	65.4% (17/26)	88.6% (31/35)	0.54
IFAT (serum)	0.64	0.45-0.83	92.3% (24/26)	74.3% (26/35)	0.67
ELISA (TgSAG1) (serum)	0.61	0.41-0.81	88.5% (23/26)	74.3% (26/35)	0.63
MajRef* (serum)	**0.80**	**0.65-0.95**	**92.3% (24/26)**	**88.6% (31/35)**	**0.81**

* References based on the test result of three tests. Once two of the tests tested positive the serum was recorded as "reference positive"

Level of antibodies relative to outcome of bioassay (Table 5)

When the outcome of the mouse bioassay (26 positive in heart and 3 in addition positive in heart) was stratified according to the different levels of antibodies (IgG) as determined by MAT, IFAT and ELISA at least in MAT and ELISA a good relationship between the level of antibody and the proportion of bioassay positive chicken was observed. In IFAT the relationship was less clear; however chickens with titres < 1:50 were rarely bioassay positive (7%, 2/28) while in all groups of chicken with positive IFAT titres about two thirds of chickens tested positive in bioassay. The highest proportion of bioassay positive chickens among tested negative (i.e. showed no titre) were in MAT.

Linear regression between the mean values for $\log_{10}$ transformed titres or ELISA indices for each IgG antibody level (Table 5) revealed correlation with R^2 values of 35.0%, 55.1%, and 70.9% for MAT, IFAT and ELISA, respectively. The proportion of hens positive in bioassay increases with the means for $\log_{10}$ transformed titres or ELISA indices in the group of hens analysed. Linear regression between the mean values for MAT titres and the proportion of chicken tested positive in bioassay.

Linear regression between the number of days after inoculation the bioassay became positive and the serum MAT titre, the serum IFAT titre, or serum TgSAG1-ELISA indices obtained for the respective hen analysed in bioassay revealed a marginal correlation result (R^2 = 6.4%, R^2=14.0%, R^2=8.0%, respectively). The time between inoculation and the onset of toxoplasmosis in mice inoculated with chicken tissue was negatively correlated with the level of antibody in the respective chicken.

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Table 5: Proportion of chickens positive in bioassay (%BA positive) relative to the level of antibody responses in the MAT, IFAT and ELISA

		Antibody level					
	Mouse bioassay	**Level 0**	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**
MAT	**Titre**	0	1	3	6	10	>10
	Mean titre of level	0	1	3	6	10	400
	Bioassay (BA) positive	7/35	2/5	5/6	3/3	5/6	4/5
	% BA positive	20%	40%	83%	100%	83%	80%
IFAT	**Titre**	0	50	100	200	400	>400
	Mean titre of level	0	50	100	200	400	3200
	Bioassay (BA) positive	2/28	7/11	7/8	7/10	2/3	1/1
	% BA positive	7%	64%	88%	70%	66.6%	100%
ELISA (TgSAG1)	**Index**	< 0.1	≥ 0.1, < 0.2	≥ 0.2, < 0.4	≥ 0.4, < 0.8	≥ 0.8, < 1.6	≥ 1.6
	Mean index of level	0.03	0.15	0.29	0.62	1.10	2.23
	Bioassay (BA) positive	1/20	2/9	3/8	2/2	9/9	9/13
	% BA positive	5%	22%	38%	100%	100%	69%

Results of Magnetic-Capture-PCR (MC-PCR) (Table 6-7)

Almost the same amount of tissue used for bioassay was also applied for MC-PCR. A mean of 3.1 g of heart or amean of 35.1 g of drumstick musculature were used for MC-PCR. In case of heart the digested tissue was completely used for magnetic capture.

In total 29 of the 61 chickens examined by MC-PCR for *T. gondii* revealed a positive result in MC-PCR. In 22 of 29 MC-PCR-positive chickens only the examination of heart revealed a positive result. In 1 of the 29 MC-PCR-positive chickens only the examination of drumstick revealed a positive result. In 5 of 29 MC-PCR-positive hens the examination of both, drumstick and heart revealed a positive result.

Agreement between serologic results and Magnetic-Capture-PCR (MC-PCR)

Linear regression between the parasite number estimates for chicken heart (MC-PCR) and the log10 transformed MAT titres, IFAT titres and TgSAG1 ELISA indices revealed relationships characterized by low R^2-values of 2.1%, 5.7% and 7.1%, respectively.

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Table 6: Summary on the characteristics of serological tests relative to MC-PCR

Test	Kappa	Kappa (95% CI)	Sensitivity	Specificity	Youden's index
MAT	0.68	0.48-0.86	69.0% (20/29)	96.9% (31/32)	0.66
IFAT	0.67	0.49-0.86	89.7% (26/29)	78.1% (25/32)	0.68
ELISA (TgSAG1)	0.77	0.62-0.94	93.1% (27/29)	84.4% (27/32)	0.77
MajRef*	***0.90***	***0.80-1.00***	***93.1% (27/29)***	***96.9% (31/32)***	**0.90**

* References based on the test result of three tests. Once two of the tests tested positive the serum was recorded as "reference positive"

ELISA: Thirty-two ELISA positive chickens were selected for bioassay and PCR (MC-PCR and conventional real-time PCR) and 29 ELISA negative were also analyzed by mouse-bioassay and PCR. 84.4% (27/32) of the ELISA-positive chicken rendered a positive MC-PCR. In contrast only 6.9% (2/29) of the ELISA-negative chicken revealed a positive MC-PCR. Sensitivity of ELISA was 93.1% (27/29) and specificity of ELISA was 84.4% (27/32). The agreement between MC-PCR and ELISA was characterized by a Kappa of 0.77 (0.62-0.94, 95% CI) (substantial concordance).

MAT: Twenty-one MAT positive chickens had been selected for mouse bioassay and PCR. In addition, 40 MAT negative chickens were also analyzed by mouse-bioassay and PCR. 95.2% (20/21) of the MAT-positive chicken rendered a positive MC-PCR. However, also 22.5% (9/40) of the MAT-negative chicken showed a positive MC-PCR. Sensitivity of MAT was 69.0% (20/29) and specificity of MAT was 96.9% (31/32). The agreement between MC-PCR and MAT was characterized by a Kappa of 0.68, (0.48-0.86, 95% CI) (substantial concordance).

IFAT: Thirty-three IFAT positive chickens had been selected for mouse bioassay and PCR. Further 28 IFAT negative chickens were also analyzed by mouse-bioassay and PCR. 78.8% (26/33) of the IFAT-positive chicken rendered a positive MC-PCR. Only 10.7% (3/28) of the IFAT-negative chicken showed a positive MC-PCR. Sensitivity of IFAT relative to MC-PCR was 89.7% (26/29) and specificity of IFAT was 78.1% (25/32). The agreement between MC-PCR and IFAT was characterized by a Kappa of 0.67 (0.49-0.86, 95% CI) (substantial concordance).

Majority: If the classification is done based on the majority of serological test results, 28 majority-positive chickens had been selected for mouse bioassay and PCR. Further 33 majority-negative chickens were also analyzed by mouse-bioassay and PCR. 96.4% (27/28) of the majority positive chicken rendered a positive MC-PCR. Only 6.1 % (2/33) of the majority negative chicken showed a positive MC-PCR. Sensitivity of the majority reference was 93.1% (27/29) and specificity of this reference was 96.9% (31/32). The agreement between MC-PCR and MajRef was characterized by a Kappa of 0.90 (0.80-1.00, 95% CI) (almost perfect concordance).

Agreement between bioassay and Magnetic-Capture-PCR (MC-PCR)

There was substantial concordance between the results of bioassay and MC-PCR which was characterized by a Kappa value of 0.77 (0.60-0.93, 95% CI). Only 2 of the bioassay positive chickens tested negative in MC-PCR. In bioassay positive chicken using heart tissue (n=24), 5 were positive in both heart and drumstick, 19 were positive only in heart and not a single hen positive only in drumstick. In those hens bioassay positive in drumstick (and at the same also bioassay positive in heart), 2 were MC-PCR positive in heart and drumstick and one positive only in heart (Table 7).

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Five bioassay negative chickens tested positive in the MC-PCR. In all of these cases the serum ELISA revealed a positive result and in 4 of 5 cases IFAT and MAT on sera revealed a positive result, too. In four of the cases only the chicken heart and in one case only the chicken drumstick tested positive in MC-PCR.

Table 7: Comparison of Bioassay and MC-PCR results

	Bioassay positive			**Bioassay negative**
	Total (Positive MC-PCR: HD, H, D*)	Heart (MC-PCR: HD, H, D*)	Drumstick** (MC-PCR: HD, H, D*)	**Total (Positive MC-PCR: HD, H, D*)**
MC-PCR positive	24 (5/19/0)	24 (5/19/0)	3 (1/2/0)	5 (0/4/1)
MC-PCR negative	2 (0/0/0)	2 (0/0/0)	0 (0/0/0)	30 (0/0/0)

* Figures in brackets represent MC-PCR positive findings in both heart and drumstick (HD), only heart (H) or only drumstick (D) of an individual animal.
** All chickens which tested bioassay positive in drumstick tissue also tested bioassay positive in heart tissue.

Results of conventional real-time PCR (cvPCR) on pepsin-digested material (Table 8-12)

Aliquots of remaining material of pepsin-digested tissues (heart, drumstick) which had not been inoculated into bioassay mice were DNA-extracted and analysed by a conventional real time PCR (cvPCR) since the use of the primers Tox-9F and Tox-11R (primer sequence in 7.1.2.3) revealed problems which could not be solved in time. A second real-time PCR protocol was applied using primers and protocol published by (Talabani et al., 2009). This PCR showed a similar analytical sensitivity as the PCR with Tox-9F and Tox-11R but no unspecific products were observed in tissues from chicken (specific products had a size of 125 bp and a locked nucleic acid probe was used in this real-time PCR). In this PCR a Cq value of > 40 was regarded as negative. In case of Cq values of 35-40 the specific size of the amplified products (125 bp) was confirmed by agarose gel electrophoresis.

Table 8: Comparison of conventional real-time PCR (cvPCR) and MC-PCR results (not stratified for tissue)

	MC-PCR positive, total	**MC-PCR negative, total**
cvPCR positive, total	27	3
cvPCR negative, total	2	29

In total 30 of the 61 chickens examined by cvPCR for *T. gondii* revealed a positive result. In 29 of 30 cvPCR-positive chickens only the examination of heart revealed a positive result. In 1 of the 30 cvPCR-positive hens the examination of both, drumstick and heart revealed a positive result.

The agreement between cvPCR and MC-PCR (results not stratified for tissue) was characterized by a Kappa value 0.84 (0.70- 0.97; almost perfect concordance). In 5 samples a discordant result was obtained in both PCRs (Table 8).

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Table 9: Comparison of conventional real-time PCR (cvPCR) and MC-PCR results (restricted to heart)

	MC-PCR positive, total	MC-PCR negative, total
cvPCR positive, total	27	3
cvPCR negative, total	1	30

The agreement between conventional real-time PCR (cvPCR) and MC-PCR (results restricted to heart) was characterized by a Kappa value of 0.87 (0.75- 0.99, almost perfect concordance). While MC-PCR detected 28 heart samples as positive, real-time PCR detected 30 as positive. In case of 4 heart samples both PCRs had discordant results (Table 9). There was a correlation between the Cq values observed in conventional real-time PCR and the parasite estimates in MC-PCR (R^2=0.46; Fig. 1) for heart samples.

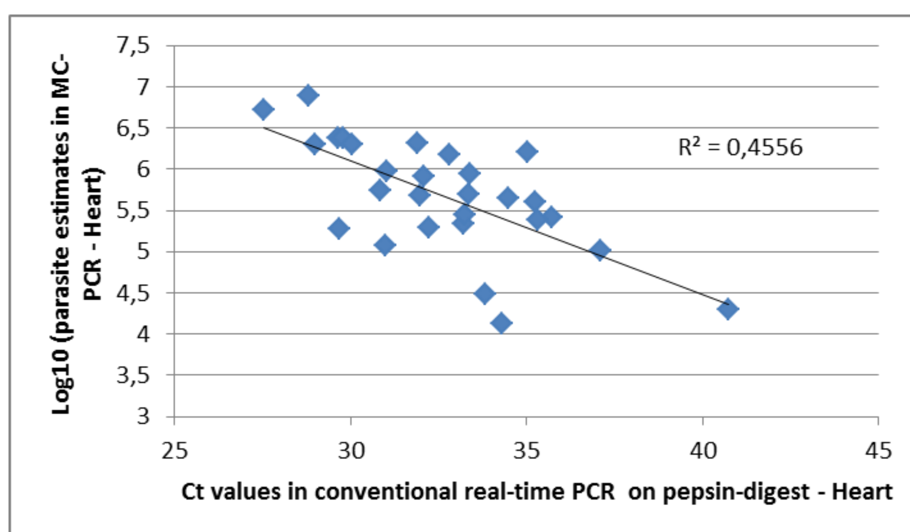

Figure 1: Linear regression between the parasite number estimates for chicken heart (MC-PCR) and the results (Ct values) obtained in conventional real-time PCR (cvPCR) on pepsin-digested heart tissues

Table 10: Comparison of conventional real-time PCR (cvPCR) and MC-PCR results (restricted to drumstick)

	MC-PCR positive, total	MC-PCR negative, total
cvPCR positive, total	1	0
cvPCR negative, total	5	55

The agreement between conventional real-time PCR and MC-PCR (results restricted to drumstick) was characterized by a Kappa value of 0.27 (-0.15- 0.68; fair concordance). While MC-PCR detected 6 of the drumstick samples as positive the cvPCR detected 1 positive sample, which was also positive in MC-PCR (Table 10).

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Table 11: Summary on the comparison of the results in serological tests and in conventional real-time PCR (cvPCR) result

Test (analyte)	Kappa	Kappa (95% CI)	Sensitivity	Specificity	Youden's index
MAT (serum)	0.64	0.45-0.82	66.7% (20/30)	96.8% (30/31)	0.63
IFAT (serum)	0.71	0.53-0.88	90.0% (27/30)	80.6% (25/31)	0.71
ELISA (TgSAG1) (serum)	0.67	0.49-0.86	86.7% (26/30)	80.6% (25/31)	0.67
MajRef* (serum)	***0.87***	***0.75-0.99***	***90.0% (27/30)***	***96.8% (30/31)***	**0.87**

* References based on the test result of three tests. Once two of the tests tested positive the serum was recorded as "reference positive

ELISA: Thirty-two ELISA positive chickens were selected for bioassay and PCR and 29 ELISA negative were also analyzed by mouse-bioassay and PCR. 81.3% (26/32) of the ELISA-positive chicken rendered a positive result by conventional real-time PCR using pepsin-digested material. In contrast only 13.8% (4/29) of the ELISA-negative chicken revealed a positive real-time PCR. Sensitivity of ELISA was 86.7% (26/30) and specificity of ELISA was 80.6% (25/31). The agreement between real-time PCR and ELISA was characterized by a Kappa of 0.67 (0.49-0.86, 95% CI) (substantial concordance).

MAT: Twenty-one MAT positive chickens had been selected for mouse bioassay and PCR. In addition, 40 MAT negative chickens were also analyzed by mouse-bioassay and PCR. 95.2% (20/21) of the MAT-positive chicken rendered a positive conventional real-time PCR. However, also 25.0% (10/40) of the MAT-negative chicken showed a positive real-time PCR. Sensitivity of MAT was 66.7% (20/30) and specificity of MAT was 96.8% (30/31). The agreement between real-time PCR and MAT was characterized by a Kappa of 0.64, (0.45-0.82, 95% CI) (substantial concordance).

IFAT: Thirty-three IFAT positive chickens had been selected for mouse bioassay and PCR. Further 28 IFAT negative chickens were also analyzed by mouse-bioassay and PCR. Of the IFAT-positive chicken, 81.8% (27/33) rendered a positive conventional real-time PCR. Only 10.7% (3/28) of the IFAT-negative chicken showed a positive real-time PCR. The sensitivity of and specificity of IFAT relative to real-time PCR were 90.0% (27/30) and 80.6% (25/31) respectively. The agreement between real-time PCR and IFAT was characterized by a Kappa of 0.71 (0.53-0.88, 95% CI) (substantial concordance).

Majority: If the classification is done based on the majority of serological test results, 28 majority-positive chickens were selected for mouse bioassay and PCR. Further 33 majority-negative chickens were also analyzed by mouse-bioassay and PCR. The majority positive chicken, 96.4% (27/28), rendered a positive real-time PCR. Only 9.1 % (3/33) of the majority negative chicken showed a positive real-time PCR. Sensitivity of the majority reference was 90.0% (27/30) and specificity of this reference was 96.8% (30/31). The agreement between real-time PCR and MajRef was characterized by a Kappa of 0.87 (0.75-0.99, 95% CI) (almost perfect concordance).

Agreement between bioassay and conventional real-time PCR (cvPCR)

There was almost perfect concordance between the results of bioassay and conventional real-time PCR on pepsin-digested material, which was characterized by a Kappa value of 0.87 (0.74-0.99, 95% CI). None of the bioassay positive chickens tested negative in cvPCR. In bioassay positive chicken using heart tissue (n=26), 1 was positive in both heart and drumstick, 25 were positive only in heart and not

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a single hen positive only in drumstick. Three hens were positive in the bioassay in drumstick (and at the same time also bioassay positive in heart), and were cvPCR positive in heart only (Table 12).

Four bioassay negative chickens tested positive by the cvPCR. In 3/4 of these cases the ELISA, the IFAT and MAT on sera revealed a positive result. The remaining chicken was negative by all serological tests. In all four cases only the chicken heart tested positive by cvPCR (Table 12).

Table 12: Comparison of bioassay and conventional real-time PCR results (cvPCR) on pepsin digested material

	Bioassay positive			**Bioassay negative**
	Total (Positive cvPCR: HD, H, D*)	Heart (MC-PCR: HD, H, D*)	Drumstick** (MC-PCR: HD, H, D*)	**Total (Positive cvPCR: HD, H, D*)**
cvPCR positive	26 (1/25/0)	26 (1/25/0)	3 (0/3/0)	4 (0/4/0)
cvPCR negative	0 (0/0/0)	0 (0/0/0)	0 (0/0/0)	31 (0/0/0)

* Figures in brackets represent cvPCR positive findings in both heart and drumstick (HD), only heart (H) or only drumstick (D) of an individual animal.
** All chicken which tested bioassay positive in drumstick tissue tested also bioassay positive in heart tissue.

Agreement between serologic results and a reference based on any direct detection method (bioassay and two PCRs) (Table 13)

As a further reference standard for the validation of the ability of serological assays to detect chickens which harbour *T. gondii* in their tissues was generated by combining the results of the bioassay and the all chicken that tested positive in at least one of the three tests were regarded as positive. The reference standard was named BA+MC+cvPCR (Bioassay + MC-PCR+ conventional real-time PCR).

Table 13: Summary on the characteristics of serological tests based on serum relative to the results in direct detection methods (BA+MC+cvPCR) as a reference

Test	**Kappa**	**Kappa (95% CI)**	**Sensitivity**	**Specificity**	**Youden's index**
MAT	0.65	0.47-0.82	65.6% (21/32)	100% (29/29)	0.66
IFAT	0.70	0.53-0.88	87.5% (28/32)	82.8% (24/29)	0.70
ELISA (TgSAG1)	0.74	0.57-0.91	87.5% (28/32)	86.2% (25/29)	0.74
MajRef*	**0.87**	**0.75-0.99**	**87.5% (28/32)**	**100% (29/29)**	**0.88**

* References based on the test result of three tests. Once two of the tests tested positive the serum was recorded as "reference positive"

ELISA: Thirty-two ELISA positive hens were selected for bioassay and PCR and 29 ELISA negative chickens were also analysed by mouse-bioassay and PCR. The ELISA-positive chicken, 87.5% (28/32), rendered a positive bioassay or PCR result. In contrast, four (13.8%; 4/29) of the ELISA-negative hens revealed a positive bioassay or PCR. The sensitivity of ELISA was 87.5% (28/32) and

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the specificity of ELISA was 86.2% (25/29) relative to the BA+MC+cvPCR reference. The agreement between BA+MC-cvPCR and ELISA was characterized by a Kappa of 0.74 (0.57-0.91, 95% CI) (substantial concordance).

MAT: Twenty-one MAT positive hens had been selected for mouse bioassay and PCR. In addition, 40 MAT negative chickens were also analysed by mouse-bioassay and PCR. 100% (21/21) of the MAT-positive hens revealed a positive bioassay or MC-PCR result. However, also 27.5% (11/40) of the MAT-negative chickens showed a positive bioassay or MC-PCR. Sensitivity of MAT was 65.6% (21/32) and specificity of MAT was 100% (29/29) relative to BA+MC-cvPCR reference. The agreement between BA+MC+cvPCR and MAT was characterized by a Kappa of 0.65 (0.47-0.82, 95% CI) (substantial concordance).

IFAT: Thirty-three IFAT positive hens had been selected for mouse bioassay and PCR. Further 28 IFAT negative chickens were also analysed by mouse-bioassay and PCR. The IFAT-positive hens, 84.8% (28/33), rendered a positive PCR or bioassay. Only 14.3% (4/28) of the IFAT-negative hens showed a positive bioassay. Sensitivity of IFAT relative to BA+MC+cvPCR reference was 87.5% (28/32) and specificity of IFAT was 82.8% (24/29). The agreement between BA+MC+cvPCR and IFAT was characterized by a Kappa of 0.70 (0.53-0.88, 95% CI) (substantial concordance).

Majority (MajRef): If the classification is done based on the majority of serological test results, 28 majority-positive chickens had been selected for mouse bioassay and PCR (reference called MajRef). Further 33 majority-negative chickens were also analyzed by mouse-bioassay and PCR. Of the MajRef positive chickens, all (28/28) rendered a positive PCR or bioassay. Only 12.1% (4/33) of the MajRef negative hens showed a positive PCR or bioassay. Sensitivity of the majority reference was 87.5% (28/32) and specificity of this reference was 100% (29/29) relative to the BA+MC+cvPCR reference. The agreement between BA+MC-cvPCR and MajRef was characterized by a Kappa of 0.87 (0.75-0.99, 95% CI) (almost perfect concordance).

Results in TgSAG1-ELISA, IFAT, MAT using chicken meat juice (collected from heart, drumstick and breast musculature) (Table 14)

Fluids from tissues used for bioassay or MC-PCR (heart muscle, drumstick muscle) and from an additional tissue (breast muscle) were collected and examined in three tests (MAT, IFAT, ELISA) for antibodies against *T. gondii*. Since previous experiences suggested that fluids contain only about 10% of the antibodies in the same volume of serum, a 10-times higher concentration of samples were employed in IFAT (dilutions starting at 1:5) and ELISA (fluid dilution 1:20). In the MAT the use of a lower test dilution was not possible but a less stringent positive cut-off for data analysis was applied (positive threshold dilution applied in MAT: 1:1).

Overall (mean) the TgSAG1-ELISA showed the best test characteristics. Overall specificity was 99% and sensitivity was 87% using tissue fluids as analyte. The agreement between BA+MC+cvPCR reference and ELISA was characterized by a Kappa of 0.83-0.87. MAT and IFAT performed similar although IFAT showed a higher specificity. MAT showed a higher sensitivity as compared to IFAT. The agreement between BA+MC+cvPCR reference and MAT was characterized by Kappa values of 0.28-0.82. The agreement between BA+MC+cvPCR reference and IFAT was characterized by a Kappa of 0.55-0.67.

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Table 14: Summary on the characteristics of tests used to examine chickens tissue fluids relative to the *T. gondii* reference BA+MC+vCqCR*

Test Analyte	**Kappa**	**Kappa (95% CI)**	**Sensitivity**	**Specificity**	**Youden's index**
MAT					
Heart	0.82	0.68-0.97	83.9% (26/31)	100% (25/25)	0.84
Drumstick	0.77	0.61-0.93	84.4% (27/32)	92.9% (26/28)	0.77
Breast	0.28	0.07 0.50	90.3% (28/31)	37.0% (10/27)	0.27
Mean	*0.62*		*86%*	*77%*	*0.63*
IFAT					
Heart	0.67	0.48-0.85	77.4% (24/31)	89.7% (26/29)	0.67
Drumstick	0.55	0.37-0.74	56.3% (18/32)	100% (29/29)	0.56
Breast	0.61	0.43- 0.79	62.5% (20/32)	100% (29/29)	0.63
Mean	*0.61*		*65%*	*97%*	*0.62*
ELISA (TgSAG1)					
Heart	0.83	0.70-0.97	87.1% (27/31)	96.6% (28/29)	0.84
Drumstick	0.87	0.75-0.99	87.5% (28/32)	100% (29/29)	0.88
Breast	0.87	0.74-0.99	87.1% (27/31)	100% (29/29)	0.87
Mean	*0.86*		*87%*	*99%*	*0.86*

* References based on combining the results of bioassay and PCR. All chicken that tested positive in at least one of the two tests were regarded as "positive", the remaining as "negative".

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4.1.3.2. Identification of potential risk factors

The main objective of the field study on laying hens was to examine the relationship between seropositivity and the presence of infective *T. gondii* tissue cysts in edible tissues of chicken. The study was restricted to laying hens because due to their age at slaughter a higher prevalence was expected as compared to broiler chicken. Consequently, the study was restricted to farms in which animals had outside access and on which no coccidiostats were used which could interfere with the infectious status of these birds. Therefore, we restricted the examination to organic farms (large farms) and backyard farms (small farms) because of the high probability of infection. Farms were not selected randomly, but by convenience.

On each farm the laying hens were selected based mainly on age but also on convenience. There are differences in the distribution of age classes of sampled animals between the farms (Table 15). The number of chicken selected for serological analysis varied between farms and ranged from 2-60 (Table 17). The sample size per farm was dependent on the farm size (Table 17, hens per unit), the availability of chicken and the willingness of the farmer to provide chicken for our study.

A total number of 19 farms were investigated. On each farm a questionnaire (Appendix 6.5.5 Poultry 5) was filled in during or shortly after sampling (Table 16). Based on the proportion of seropositive hens on the farm, the 19 farms were stratified in farms with a high prevalence (HP-farms) and in farms with a zero or low proportion of positive hens (LP-farms). A hen was regarded as seropositive if the TgSAG1-ELISA showed a positive test result (Table 17). A farm was regarded as positive if at least 20% of the hens sampled per farm had tested positive to avoid an overestimation of the number of positive farms due to a putative lack of specificity of the TgSAG1.

A total number of 35 variables were recorded (Table 16). In 11 variables the answers were very heterogeneous and had to be categorized prior to analysis (Table 16). For the answers regarding type of bedding, on disinfection of stable, hygienic measures on the chicken-run a meaningful categorization was not possible and the answers on these variables were removed from statistical analysis.

Table 15: Age of chicken stratified by origin

	Farm type		
Age category	***Large (>500)***	***Small (<50)***	***Total***
1 (10-12 months)	35 (9%)	22 (26%)	57 (12%)
2 (>12- 24 months)	349 (91%)	18 (21%)	367 (78%)
3 (>24 months)	0 (0%)	20 (23%)	20 (4%)
NA	0 (0%)	26 (30%)	26 (6%)
Total	384 (100%)	86 (100%)	470 (100%)

In a univariate statistical analysis it was assessed whether the serological status of each of the farms could be modelled by Chi-square using questionnaire data as explaining variables. Variables related to definitive host (CatPremisesPresence, CatPremisesPresenceNo, CatsForRodCont) revealed as risk factors. Other putative risk factors related to the farm size (FarmSize) were SizeStableC,

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SqmPerAnimalChRunC. The existence of an empty period (EmptyPeriod) or a long cleaning interval for the hen house or hall (FreqCleaningC) tested as putatively protective variables. A low hen density on the chicken-run (MoreOr10sqm; i.e., 10 squared meter (sqm) or more per hen) turned out as a putative risk factor (Table 18).

4.1.4. Discussion and conclusion

From a total of 19 farms tested, 61 laying hens were selected from 16 farms for the further analyses to compare the concordance between seropositivity and the presence of viable tissue cysts or positivity in both PCR's (MC-PCR and conventional real-time PCR on pepsin-digested tissues). Hens from two farms were not used because enough negative or inconclusive ELISA hens had already selected from farms enrolled previously.

Farm seropositivity and isolation of T. gondii via bioassay

Isolation in bioassay was achieved after pepsin-digestion. Since the survival time of tachyzoites has been postulated to be short during pepsin-digestion, the isolation of *T. gondii* after pepsin digest is a clear indication for the presence of tissue cysts in the musculature of these chickens. Thus, the tissues of the respective hens had to be regarded as potentially infectious for humans.

Isolation after a positive mouse bioassay was always achieved in more than a single hen sampled per farm. This suggests a high prevalence of *T. gondii* on the respective farms. Isolation was achieved also from seronegative hens. However, these seronegative chickens always originated from farms, where also seropositive hens had been detected.

Isolation was only achieved in hens purchased on small backyard farms (8/12 small farms). In none of the large commercial organic farms (0/7 large farms) an isolation was achieved. This suggests that the parasite load in backyard hens seems to be higher out of several potential reasons: an often higher age of the hens, a closer contact between hens and definitive hosts (i.e. domestic cats), a less hygienic environment, etc. Potential reasons were covered by a questionnaire and the respective results are going to be discussed later-on.

Isolation was more likely to occur in hens with stronger antibody response as shown by linear regression. In addition, during mouse bioassay experiments it was observed that mice inoculated with pepsin-digests from hens with higher antibody-response developed earlier toxoplasmosis than other mice. This was independent of the serological assay used, but more pronounced when serological results had been determined by ELISA. These results suggest that the level of antibody response is partially associated with the parasitic load in tissues.

Farm seropositivity and detection of T. gondii via MC-PCR or conventional real-time PCR (cvPCR) on pepsin-digested tissues

PCR detection of *T. gondii* is not a direct indicator for the presence of tissue cysts (containing bradyzoites) because also the DNA of tachyzoites is going to be detected by this method. However, under the assumption of chronic infection dominating in the majority of hens selected for the present study, a detection of parasitic DNA may correlate well with the presence of tissue cysts in the animals. Our observation is in agreement with this assumption: the results of bioassay correlated well with the results of MC-PCR (Kappa 0.77) and conventional real-time PCR (cvPCR) on heart tissue (Kappa 0.87). In all farms in which bioassay positive hens had been identified also MC-PCR or cvPCR positive hens were detected. There were two exceptions: i. MC-PCR detected *T. gondii*-DNA in the

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heart tissue of a serologically positive hen from a large farm while mouse bioassay had revealed a negative result. ii. cvPCR detected *T. gondii*-DNA in the heart tissue of a serologically negative hen from another large farm while mouse bioassay had revealed a negative result.

Relationship between seropositivity in chickens and presence of T. gondii in meat

Seropositivity was determined in serum of laying hens using three tests, MAT, IFAT and an ELISA based on TgSAG1, a major immunodominant tachyzoite surface antigen. To assess the relationship of seropositivity and the presence of *T. gondii* all hens which either tested positive in mouse bioassay or in MC-PCR were regarded as true positive (i.e. as reference standard, designated BA+MC reference).

Kappa values characterizing the agreement between mouse bioassay or MC-PCR and serological tests to predict positivity in bioassay ranged from 0.67-0.77 (substantial concordance) with the ELISA showing the highest and MAT the lowest value. This was caused by a low sensitivity of MAT relative to the reference standard. Youdens's index was used to characterize the diagnostic potential of the different assays. Youdens's index ranged from 0.68-0.77 (Table 11) with the lowest value observed in MAT and the highest in ELISA.

In general, on farms with a high seroprevalence in hens at the end of laying period, there was a high probability of identifying positive chickens either by bioassay or MC-PCR. Although none of the serological tests was perfect in detecting bioassay or MC-PCR positive animals, all serological tests detected seropositive hens on those farms at which bioassay or MC-PCR positive hens had been detected. Thus, results of our study suggest that serology is able to identify farms with *T. gondii* tissue cyst positive hens.

Relationship between positive antibody detection in meat juices in chickens and presence of T. gondii in meat

Meat juices (meat juices from heart, breast musculature or drumstick) were also tested in MAT, IFAT and ELISA. The IFAT and ELISA test procedures were adapted to the expected 10-times lower antibody concentration in meat juices and the same cut-offs as for serum were used in these tests. In MAT the test procedure was not changed but as a positive cut-off 1:1 (fluids) was used instead of 1:6 (serum).

Results of antibody detection in meat juices were compared with the BA+MC reference (chickens either positive in bioassay or MC-PCR). The Kappa values characterizing the agreement between BA+MC+cvPCR and the specific detection of IgG antibodies in meat juices ranged from 0.28 (fair concordance) to 0.87 (almost perfect concordance), with the ELISA showing the highest and the MAT the lowest mean value. Overall the mean YI ranged from 0.62-0.86 with the ELISA having the highest (0.86) and the MAT the lowest mean value (0.62). MAT and IFAT performed optimal when fluid collected from heart muscle was analyzed for specific antibodies. In case of the ELISA all fluids worked fine but the highest Kappa value was observed for fluid collected from drumstick muscle.

Presence and viability of T. gondii in heart and drumstick, both edible organs of chicken

In all direct detection methods (bioassay, MC-PCR, conventional real-time PCR) positive heart tissues dominated (in 31/32 hens) which confirms the results of the extensive literature review. Although obviously not a predilection site, drumstick tissues were found positive in about one quarter of the laying hens (in 8/32 hens) in at least one of the three direct detection methods mentioned above. All chicken except one were negative in drumstick although positive in heart tissues when analyzed by

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conventional real-time PCR suggesting a lower parasite load in drumstick tissue as compared to heart. Both tissues, heart and drumstick, are consumed. However, in case of laying hens (which are most likely not used for barbecue) both tissues are usually consumed after a heat treatment most likely sufficient to kill *T. gondii*.

But altogether, infection of organic raised hens accompanied with the presence of infectious *T. gondii* tissue cysts in tissues of these hens, is not a rare event. Therefore, there is a potential risk consuming undercooked meat of laying hens.

Identification of potential risk factors for T. gondii infection in laying hens from organic farming

In large farms the majority of laying hens belonged to age class 2 (12-24 months old hens). In small farms all age categories had been sampled with a large proportion of animals belonging to age class 3 (older than 24 months). Because with increasing age the chance of seropositivity increased, the age related-difference between the farms may have caused - at least - partially the differences in prevalences between large and small farms. However, also age class 1 (10-12 months old) was overrepresented in small farms. Therefore, results of this non-representative study have to be interpreted with care.

Nevertheless, management practices and other farm characteristics of small farms may have contributed to the differences observed and could indeed represent potential risk factors. Among these variables there were variables related to the definitive host of *T. gondii*, i.e. the presence of cats on the premises and the number of cats present on premises. In addition, the use of cats for rodent control turned out as a putative risk factor. Other variables, which also could explain a better transmission of *T. gondii* on farms, are differences in chicken density on positive farms. A high chicken density – as it was reported by the hen owners on large farms – may prevent cats from entering the chicken-run and thus reduce the chance of a contamination of soil or ground with oocysts. A low chicken density on small farms may allow the individual chicken the access to a larger area which in addition may favour the chance of acquiring infection. The existence of an empty period was another characteristic for large farms. A protective effect of this variable could be explained by the assumption that an empty period allows the farmer to clean the hen house/hall more thoroughly. A better cleaning may remove potential *T. gondii* oocyst contaminations. Other variables, like farm size and size of premises (SizeStableC) or a long cleaning interval for the hen house or hall (FreqCleaningC) which was typical for large farms are difficult to explain by the available knowledge on *T. gondii* biology. These variables may thus represent confounding variables.

In summary, the identification of cat-related variables as putative risk factors confirms previous findings for other farm animal species in the extensive literature review. Measures to prevent *T. gondii* infection in chicken should include biosafety protocols focusing on keeping farm premises free of cats. The use of cats for rodent control is not recommended. On the other hand, generally low *T. gondii* prevalences were observed on all large farms, even when cats were present on farm. This shows that under conditions of large organic farms the chance is very low that *T. gondii* is able to enter hens reared in this type of farms. Hens from small farms were regularly positive; chicken meat from these farms should be consumed only after a sufficient heat treatment to avoid *T. gondii* transmission and the transmission of other pathogens.

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Conclusions on field study in chickens:

1. There is substantial concordance between mouse bioassay and MC-PCR in chickens (i.e. laying hens) selected and analyzed in the field study.
2. In field hens the vast majority of positive findings by mouse bioassay, by MC-PCR as well as by conventional real-time PCR were in heart. Drumstick musculature as another representative of an edible tissue was less frequently positive in mouse bioassay and MC-PCR (about one quarter of the musculature was positive).
3. Under field conditions serological tests performed well in identifying mouse-bioassay, MC-PCR, or conventional real-time PCR positive chickens. IFAT and TgSAG1-ELISA showed a combination of both, high sensitivity and specificity to detect bioassay, MC-PCR, or conventional real-time PCR positive hens. Examination of meat juices from breast, drumstick or heart musculature revealed similar results in IFAT and TgSAG1-ELISA.
4. A positive bioassay was more likely to occur in hens with stronger antibody response as shown by linear regression.
5. A non-representative analysis of field data suggests that under intensive management conditions on large organic farms which provide outdoor access for hens, the prevalence of TgSAG1-ELISA positive laying hens is low. No bioassay positive hen was identified on large organic farms. Only a single chicken from a large farm revealed a MC-PCR positive result. In addition, another single hen from a large farm revealed a positive results in conventional real-time PCR.
6. Variables related to the presence of domestic cats on farm, the use of cats for rodent control, a low chicken density on the chicken run and other variables linked to the small size of the farm were identified as potential risk factors.
7. The aim of this study was to characterize the relationship between seroprevalence in the main livestock species and presence of *T. gondii* in meat. The study was restricted to laying hens and allows no conclusions on the correlation in broilers.
8. Serological tests are able to identify farms with viable *T. gondii* infections in hens.

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Table 16: Variables covered by a questionnaire on free-range chicken farms

Variable	Categorization of variables	Categories
Number of hens per unit	Farm size <50 hens per unit	Small: <50 hens per unit; Large: ≥ 500 per unit
Presence of cats on premises		
Number of cats presence on premises*	Number of cats presence on premises > 0	>0; 0
Presence of cats in chicken run		
Number of cats present in chicken run*	Number of cats present in chicken run >0	>0; 0
Presence of cats in hen house or hall		
Number of cats present in hen house or hall*	Number of cats present in hen house or hall >0	>0; 0
Hen house/hall cat proof		
Hen house/hall rodent proof		
Chicken-run cat-proof		
Chicken-run rodent-proof		
Ground of hen house/hall*	Soil as ground of hen house/hall	Partially or completely soil; Beton or wood, without soil
Type of bedding in hen house/hall		
Size of hen house/hall*	Size of hen house/hall ≥ 100 sqm	Small: <100 sqm; Large: ≥ 100 sqm
Hygienic measures in hen house/hall**		
Frequency of changing bedding*	Frequency of changing bedding > once per 4 months	More than once per 4 months; Less than once per 4 months or once per 4 months
Frequency of cleaning hen house/hall*	Frequency of cleaning hen house/hall > once per year	More than once a year; Less than once a year or once a year
Disinfection of hen house/hall		
Name of disinfectant(s)		
Empty period for hen house/hall		
Duration of the emplty period		
Ground in chicken-run*	Ground in chicken-run partially soil	Partially soil; Grass
Vegetation in chicken-run*	Vegetation in chicken-run	Yes; Partially
Size of chicken run in sqm*	Size of chicken run in sqm ≥ 10 sqm	≥ 10 sqm; < 10 sqm
Hygienic measures in chicken-run		
Move hens to other chicken-run		
Treatment of chicken-run with quicklime		
Other hygienic measures on chicken run*		
Period of time chicken are on run*		
Cat proof storage of fodder		
Rodent proof storage of fodder		
Cleaning of silo		
Poison is used for rodent control		
Cats are used for rodent control		
Frequency of rodent control*		

*Variables for which no statistical analysis was performed due to the large heterogeneity of answers; ** No statistics because all farms had the same answer.

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Table 17: Selected farm characteristics based on a questionnaire conducted on 19 laying hen farms on which chicken were reared free range. Seroprevalence was determined using the results of a TgSAG1-ELISA.

Farm	Seroprevalence in TgSAG1 ELISA	Positive[a]	Hens per unit	Farm Size[a]	Estimated no. of cats present on premises per week	Estimated no. of cats present on chicken run per week	Estimated no. of cats having access to stable per week	Ground in stable - details	Ground Stable[a]	Bedding	Size of stable [sqm]	Stable size[a]	Duration of empty period [weeks]	Size of chicken run [sqm]	Sqm of chicken run per animal	Hygienic measures	Hygienic measures - details[b]	Storage cat proof	Storage rodent proof	Cleaning silo	Poison for rodent control	Cats for rodent control
1	0,00	LP	3000	Large	NA	NA	0	Beton	BetonWood WithoutSoil	Wood shavings	1920	Large	5	12K	4,0	no	no	yes	yes	yes	yes	no
2**	0,05	LP	1260	Large	0	NA	0	Beton paving	BetonWood WithoutSoil	Straw	220	Large	3	7.2K	5,7	yes	Ch,Q, Cu	yes	yes	yes	yes	no
3*	0,81	HP	60	Small	NA	NA	NA	Beton	BetonWood WithoutSoil	Straw	15	Small	24	300	5,0	yes	Ch	yes	yes	NA	yes	yes
4	0,00	LP	22	Small	NA	NA	0	Soil, Beton	PartiallyOr CompletelySoil	No, straw only in nest	12	Small	ND	200	9,1	no	no	NA	no	NA	no	no
5*	0,80	HP	25	Small	2	2	0	Beton	BetonWood WithoutSoil	Straw	15	Small	ND	500	20,0	yes	Ch,R a	yes	yes	NA	no	yes
6*	1,00	HP	17	Small	6	6	6	Beton	BetonWood WithoutSoil	Straw	20	Small	ND	1.5K	88,2	no	M	no	no	NA	no	yes
7	0,00	LP	1800	Large	5	5	0	Soil, Grass	PartiallyOr CompletelySoil	Straw	188	Large	4	NA	NA	yes	Ch,C u	yes	yes	no	yes	no
8*	0,60	HP	6	Small	2	2	2	Soil	PartiallyOr CompletelySoil	Straw, hay	4	Small	8	200	33,3	no	no	yes	yes	NA	no	no
9	0,30	HP	50	Small	3	NA	NA	Beton, Wood	BetonWood WithoutSoil	Straw	30	Small	ND	500	10,0	yes	Ch,Q, Cu	yes	NA	NA	yes	no
10	0,25	HP	20	Small	1	1	0	Beton	BetonWood WithoutSoil	Straw	20	Small	ND	80	4,0	yes	Cu	yes	yes	NA	no	yes
11*	1,00	HP	15	Small	2	2	0	Beton	BetonWood WithoutSoil	Straw	25	Small	ND	150	10,0	no	no	yes	yes	no	yes	NA
12*	0,50	HP	20	Small	1	1	0	Beton	BetonWood WithoutSoil	Hay	20	Small	ND	100	5,0	yes	Cu	yes	yes	NA	no	yes
13*	1,00	HP	20	Small	1	1	1	Beton	BetonWood WithoutSoil	Straw	15	Small	ND	500	25,0	yes	Ch,Q, Cu	yes	yes	yes	yes	yes
14*	1,00	HP	15	Small	2	2	0	Beton	BetonWood WithoutSoil	Straw	20	Small	ND	200	13,3	yes	Cu	yes	yes	NA	yes	yes
15	0,33	HP	15	Small	2	2	1	Beton paving	BetonWood WithoutSoil	Straw, wood shavings	24	Small	ND	1.0K	66,7	yes	Cu	yes	yes	NA	yes	yes
16	0,00	LP	3000	Large	0	NA	NA	Beton	BetonWood WithoutSoil	Wood shavings	2250	Large	5-6	12K	4,0	yes	Re	yes	yes	yes	yes	no
17	0,08	LP	2800	Large	0	NA	NA	Beton	BetonWood WithoutSoil	Straw	470	Large	4	11K	3,9	no	no	yes	NA	no	NA	no

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Farm	Seroprevalence in TgSAG1 ELISA	Positive[a]	Hens per unit	Farm Size[a]	Estimated no. of cats present on premises per week	Estimated no. of cats present on chicken run per week	Estimated no. of cats having access to stable per week	Ground in stable - details	Ground Stable[a]	Bedding	Size of stable [sqm]	Stable size[a]	Duration of empty period [weeks]	Size of chicken run [sqm]	Sqm of chicken run per animal	Hygienic measures	Hygienic measures - details[b]	Storage cat proof	Storage rodent proof[a]	Cleaning silo	Poison for rodent control	Cats for rodent control
18	0,15	LP	1980	Large	NA	NA	NA	Beton	BetonWood WithoutSoil	Straw	363	Large	3	84.7K	4,3	yes	Cu	yes	yes	yes	yes	no
19	0,12	LP	5000	Large	0	0	0	Beton	BetonWood WithoutSoil	Staw, wood shavings, soft cell	1056	Large	5-6	25K	5,0	yes	Q	yes	yes	yes	yes	no

NA: no answer /do not know; a Categorization of answers used for statistical analysis; b Hygienic measures – details: Ch, change; Q, quicklime; Cu, Cultivation; M, Mowing; R, Refilling; * Bioassay and MC-PCR positive; ** Solely MC-PCR positive.

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Table 18: Results of a risk factor analysis (univariate, Chi square, Fisher exact) conducted on 19 laying hen farms on which chicken were reared free range. Farms had not been randomly selected but by convenience. Hens on farm were not randomly selected but based on being at the end of their laying period (≥ 10 months). A farm was regarded positive once more than 20% of the selected hens tested serologically positive in TgSAG1 ELISA. Questionnaire was conducted for a further characterization of the farms and for the generation of hypotheses on potential routes by which laying hens dad become infected by *T. gondii*.

Parameter	Category	All farms	Positive farms		OR	95%-CI Upper, Lower	P-value, 2-tailed Fisher exact
		No.	No.	%			
Farm size	**<50 hens per unit**	**12**	**11**	**91.7**	**Inf**	**4.99, Inf**	**0**
	≥ 500 per unit	**7**	**0**	**0**			
Presence of cats on premises	**Yes**	**12**	**11**	**91.7**	**Inf**	**3.32, Inf**	**0.001**
(based on yes/no anwers of farmers)	**No**	**5**	**0**	**0**			
Number of cats presence on premises	**>0**	**11**	**10**	**90.9**	**Inf**	**2.25, Inf**	**0.004**
(based on cat numbers reported by farmers)	**0**	**4**	**0**	**0**			
Presence of cats in chicken run	Yes	17	11	64.7	Inf	0.04, Inf	NS
	No	1	0	0			
Number of cats present in chicken run	>0	10	9	90	Inf	0.12, Inf	NS
	0	1	0	0			
Presence of cats in hen house or hall	Yes	11	6	54.5	Inf	0.07, 6.4	NS
	No	8	5	62.5			
Number of cats present in hen house or hall	>0	4	4	100	Inf	0.4, Inf	NS
	0	10	5	50			
Hen house/hall cat proof	Yes	9	6	66.7	2	0.23, 19.39	NS
	No	10	5	50			

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Parameter	Category	All farms	Positive farms		OR	95%-CI	P-value, 2-tailed
Hen house/hall rodent proof	Yes	1	1	100	Inf	0.03, Inf	NS
	No	14	6	42.9			
Chicken-run cat-proof	Yes	0	0	0	ND	ND	ND
	No	18	10	55.6			
Chicken-run rodent-proof	Yes	1	1	100	Inf	0.02, Inf	NS
	No	16	8	50			
Soil as ground of hen house/hall	Yes	3	1	33.3	0.3	0, 7.41	NS
	No	16	10	62.5			
Size of hen house/hall ≥ 100 sqm	**Yes**	**12**	**11**	**91.7**	**Inf**	**4.99, Inf**	**0**
	No	**7**	**0**	**0**			
Hygienic measures in hen house/hall	Yes	19	8	42.1	ND	ND	ND
	No	0	0	0			
Frequency of changing bedding > once per 4 months	Yes	8	6	75	7.5	0.52, 128.01	NS
	No	7	2	28.6			
Frequency of cleaning hen house/hall > once per year	**Yes**	**6**	**6**	**100**	**Inf**	**4.2, Inf**	**0.001**
	No	**7**	**0**	**0**			
Disinfection of hen house/hall	Yes	9	2	66.7	0.62	0.01, 17.23	NS
	No	3	5	55.6			
Empty period for hen house/hall	**Yes**	**9**	**2**	**22.2**	**0.03**	**0, 0.57**	**0.005**
	No	**10**	**9**	**90**			
Ground in chicken-run partially soil versus grass	Yes	12	6	50	0.4	0.03, 4	NS
	No	7	5	71.4			
Vegetation in chicken-run	Yes	7	5	71.4	2.5	0.25, 34.73	NS
	Partially	12	6	50			

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Parameter	Category	All farms	Positive farms		OR	95%-CI	P-value, 2-tailed
Size of chicken run in sqm ≥ 10 sqm	**Yes**	**8**	**8**	**100**	**Inf**	**2, Inf**	**0.004**
	No	**10**	**3**	**30**			
Hygienic measures in chicken-run	Yes	13	8	61.5	1.6	0.15, 17.02	NS
	No	6	3	50			
Move hens to other chicken-run	Yes	13	7	53.8	1.71	0.16, 24.78	NS
	No	6	4	66.7			
Treatment of chicken-run with quicklime	Yes	4	2	50	0.67	0.04, 11.86	NS
	No	15	9	60			
Cat proof storage of fodder	Yes	17	10	58.8	0	0, 61.23	NS
	No	1	1	100			
Rodent proof storage of fodder	Yes	15	9	60	1.5	0.02, 130.49	NS
	No	2	1	50			
Cleaning of silo	Yes	6	1	16.7	0.4	0, 46.97	NS
	No	3	1	33.3			
Poison is used for rodent control	Yes	12	6	50	0.2	0, 2.89	NS
	No	6	5	83.3			
Cats are used for rodent control	**Yes**	**8**	**8**	**100**	**Inf**	**2.97, Inf**	**0.001**
	No	**10**	**2**	**20**			

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4.2. Anatomical distribution of *T. gondii* in experimental infected chickens and turkeys

4.2.1. Introduction

The anatomical distribution of *T. gondii* tissue stages in poultry (chickens, turkeys) has not been sufficiently investigated so far though it is known that poultry often harbours *T. gondii*. However, this knowledge is essential to give recommendations about the type of tissue most suitable for routine testing for *T. gondii* presence e.g. in slaughterhouses.

Aim of the study:
Experiments were performed aiming (i) at detection of parasite DNA via magnetic capture quantitative PCR (MC-PCR) in various tissues to determine the anatomical distribution and (ii) at the antibody detection in serum and meat juice samples from various types of muscle tissues to characterize the relation between direct parasite detection and antibody detection.

As tests to detect IgG antibodies in serum or body fluids we used the modified agglutination test (MAT), a well-established in-house immunofluorescence test (IFAT) and an ELISA based on TgSAG1 (p30-ELISA (Maksimov et al., 2011)) as proposed by the European Food Safety Authority for the analysis of swine (EFSA, 2011).

4.2.2. Material and Methods

4.2.2.1. Experimental infections in chickens

We planned to examine serum samples and tissues comprising edible and non-edible organs of experimentally infected chickens (laying hens, n = 66 plus controls) using MC-PCR. These chicken tissue samples had been generated during a previous research project (Toxonet02, funded by the German Ministry of Education and Research, grant no. 01KI1002C, funding period June 2009 until June 2013) but could not be intensively examined due to funding limitations.

Most of the birds have been experimentally infected by oral inoculation with tissue cysts (n = 18) or oocysts (n = 42), thereby mimicking the natural route of infection. For the infection type II or type III strains of *T. gondii* have been used, which are common in Europe (e.g. NED, Me49, or a field strain from Czech Republic). A few chickens were inoculated intravenously with *T. gondii* tachyzoites (n=12, NED strain). Infected and control chickens were slaughtered 5 or 10 weeks post infection, representing a fattening period similar to that applied in chicken industry.

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Table 1: Number of chickens used in experiments (controls, infected, infectious stage, strain)

		Observation period (weeks p.i.)		
Infection dose and stage	**Strain**	**10**	**5**	**Total**
Controls		6	18	24
1x10^3 oocysts	field strain (CZ) ME49 NED		6 6 5	6 6 5
Subtotal 1x10^3 oocysts			*17*	*17*
1x10^5 oocysts	field strain (CZ) ME49		6 6	6 6
Subtotal 1x10^5 oocysts			*12*	*12*
1x10^6 oocysts	field strain (CZ) ME49		6 4	6 4
Subtotal 1x10^6 oocysts			*10*	*10*
1x10^6 tachyzoites	NED	6	6	12
Subtotal 1x10^6 tachyzoites		*6*	*6*	*12*
Tissue cysts (1 mouse brain)	field strain (CZ) ME49 NED		6 5 5	6 5 5
Subtotal tissue cysts (1 mouse brain)			*16*	*16*
Total		12	79	91

4.2.2.2. Experimental infections in turkeys

Turkeys were experimentally infected in a similar way as chickens (mentioned above). Similar to chickens, animals were orally infected by oocysts and tissue cysts, i.e. mimicking natural routes of infection, and a few turkeys were infected by tachyzoites. Again *T. gondii* strains were used representing common European genotypes (type II or III). According to chicken trials, frozen edible and non-edible organs as well as sera of experimentally infected turkeys (n = 39) of a previous project (Toxonet02) were made available for this EFSA project. Tissue samples were examined by MC-PCR to allow conclusions on the parasite burden and the anatomical distribution in *T. gondii* infected turkeys.

TgSAG1-ELISA for chickens and turkeys: See **protocol in the Appendices (Appendix 6.5.3 Poultry 3).**

IFAT for chickens and turkeys: **See protocol in the Appendices (Appendix 6.5.4 Poultry 4).**

Modified agglutination test (MAT): **MAT was performed as described in the Appendix 6.1.3.** The positive threshold titre in serum analysis was 1:6. For the analysis of tissue fluids of chicken examined in bioassay and MC-PCR a titre of 1:1 was selected as positive threshold titre.

Magnetic capture PCR: **See protocol in the Appendices (Appendix 6.5.2 Poultry 2).** Magnetic capture PCR was partially performed with equipment different from the equipment described in the original literature. Consequently the protocol had to be adapted.

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Table 2: Number of turkeys used in experiments (controls, infected, infectious stage, strain)

		Observation period (weeks p.i.)				
Infection dose and stage	**Strain**	**4**	**8**	**12**	**16**	**Total**
Controls			11		6	17
$1x10^3$ oocysts	field strain (CZ)		6			6
	ME49		6			6
	NED		6			6
Subtotal $1x10^3$ oocysts			*18*			*18*
$1x10^5$ oocysts	field strain (CZ)		6			6
	ME49		6			6
Subtotal $1x10^5$ oocysts			*12*			*12*
$1x10^6$ oocysts	field strain (CZ)		6			6
	ME49		4			4
Subtotal $1x10^6$ oocysts			*10*			*10*
$1x10^6$ tachyzoites	NED	6	6	6	6	24
Subtotal $1x10^6$ tachyzoites		*6*	*6*	*6*	*6*	*24*
Cysts (1 mouse brain)	field strain (CZ)		6			6
	ME49		6			6
	NED		6			6
Subtotal cysts (1 mouse brain)			*18*			*18*
Total		6	75	6	12	99

4.2.3. Results

4.2.3.1. Results in experimentally infected chickens

Serological response after experimental infection in chickens

Chickens (laying hens) were experimentally infected with tissue cysts (oral, one infected mouse brain), oocysts (10^3-10^6) or tachyzoites (intravenously, 10^6). Chickens were slaughtered either 5 weeks post infection (p.i.) or 10 weeks p.i. (in case of one group of tachyzoite-infected chickens) (Table 1).

The serological response upon infection was determined using three serological tests, MAT, IFAT, TgSAG1 ELISA.

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Table 3: Sensitivity and specificity of serological tests to detect *T. gondii* infection in experimentally inoculated chickens

Serological test	Specificity	Sensitivity			
		Tissue cyst	**Oocyst**	**Tachyzoite**	**Total**
MAT	100% (24/24)	87.5% (14/16)	97.4% (38/39)	83.3% (10/12)	92.5% (62/67)
IFAT	100% (24/24)	93.8% (15/16)	100.0% (39/39)	91.7% (11/12)	97.0% (65/67)
ELISA (TgSAG1)	100% (24/24)	87.5% (14/16)	97.4% (38/39)	75.0% (9/12)	91.0% (61/67)

All tests showed 100% specificity in non-inoculated control animals. The sensitivity of detecting experimentally inoculated chicken varied between 75% (tachyzoites infection) and 100 % (oocyst infection). The highest total sensitivity was observed in IFAT, followed by MAT. The ELISA showed a slightly lower sensitivity than MAT but still higher than 90%.

In all tests the inoculation of tachyzoites – i.e. an infectious stage most likely not causing infection in chicken under natural conditions – revealed the lowest proportion of seropositive chickens ranging from 75.0% in ELISA to 83.3% in IFAT.

No effect on test sensitivity by infectious dose was observed.

Detection of T. gondii by MC-PCR after experimental infection in chickens

MC-PCR was applied to five different tissues (brain, heart, breast muscle, thigh, drumstick) collected from the experimentally inoculated animals 5 to 10 weeks post inoculation.

Table 4: Sensitivity and specificity of MC-PCR to detect *T. gondii* DNA in tissues of experimentally inoculated chickens

MC-PCR	Specificity	Sensitivity			
		Tissue cyst	**Oocyst**	**Tachyzoite**	**Total**
Brain	100% (24/24)	56.3% (9/16)	79.5% (31/39)	0.0% (0/12)	59.7% (40/67)
Heart	100% (24/24)	56.3% (9/16)	87.2% (34/39)	0.0% (0/12)	64.2% (43/67)
Breast	100% (24/24)	25.0% (4/16)	53.8% (21/39)	0.0% (0/12)	37.3% (25/67)
Thigh	100% (24/24)	25.0% (4/16)	61.5% (24/39)	0.0% (0/12)	41.8% (28/67)
Drumstick	100% (24/24)	25.0% (4/16)	52.6% (20/38)	0.0% (0/12)	36.4% (24/66)
All tissues	**100% (24/24)**	**68.8% (11/16)**	**89.7% (35/39)**	**0.0% (0/12)**	**68.7% (46/67)**

The amount of tissue used for the different organs varied according to the size of the organ. Although only a mean of 0.69 g or 2.63 g of brain or heart were used for "sequence capture", these were the organs that tested positive in 59.7% or 64.2% of the inoculated chicken, respectively. In case of breast

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muscle, thigh and drumstick a mean of 91.4 g, 64.3 g or 54.1 g of musculature were lysed and finally a volume representing 4 g of tissue used for "sequence capture". In these tissues only 37.3%, 41.8% or 36.4% of the chicken tested positive in MC-PCR, respectively. Of all inoculated chickens, 68.7% revealed a MC-PCR positive result, independent of the type of tissue analysed.

The MC-PCR was 100% specific when applied to tissues from non-inoculated controls (i.e. all samples tested negative) (Table 4).

The sensitivity of the MC-PCR was clearly dependent from the type of parasitic stage (tachyzoites, oocyst) used to inoculate the chickens. While MC-PCR detected no parasite DNA in chickens inoculated with tachyzoites, those inoculated with oocysts revealed a positive result in almost 9 of 10 chickens (89.7%). About two thirds (68.8%) of the chicken inoculated with brains of chronically infected mice revealed a positive MC-PCR result.

Independent of the type of inoculum the tissues were ranked according to the detectability of *T. gondii* by MC-PCR (reflection anatomical distribution): heart > brain > thigh > breast muscle > drumstick (Table 4).

The number of parasites in the analyte was estimated using standard concentrations. Differences between heart or brain versus all remaining tissues were statistically significant (Dunn's method; $P<0.05$). However, there were no statistically significant differences in the estimated parasite concentrations between heart and brain. The same was the case between breast, thigh and drumstick (Fig. 1).

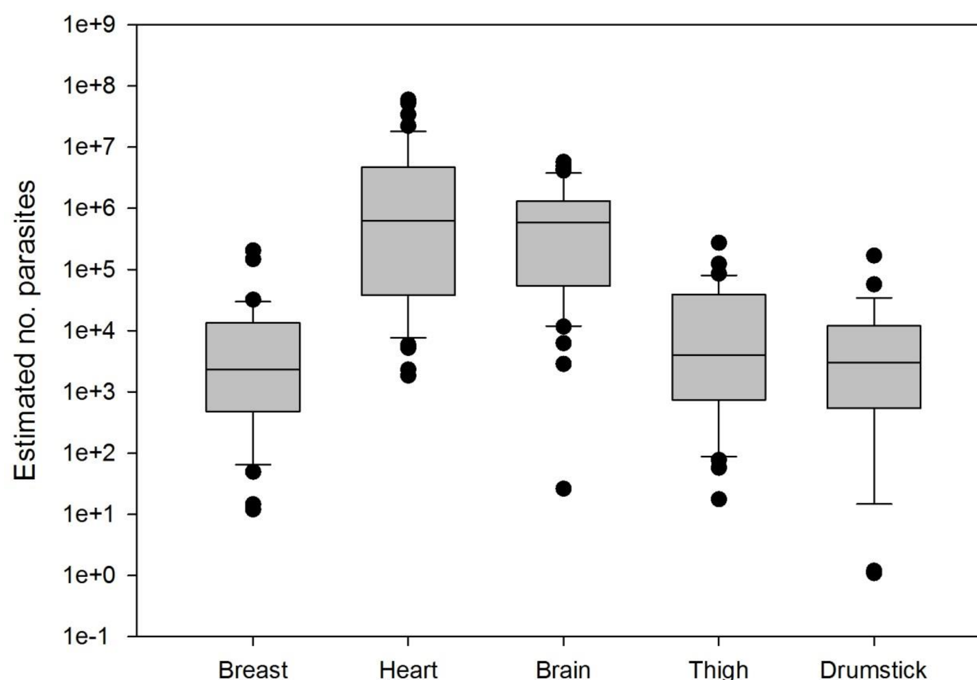

Figure 1: MC-PCR results on experimentally *T. gondii*-inoculated chickens. Estimated number of parasitic stages in samples analyzed. Differences between heart or brain versus all remaining tissues were statistically significant (Dunn's method; $P< 0.05$).

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Diagnostic characteristics of serological tests to identify MC-PCR T. gondii positive chickens after experimental infection using serum or body fluids

For the examination of diagnostic characteristics of different antibody detection techniques applied to serum and various body fluids (heart, breast muscle, thigh, drumstick) the results of MC-PCR were used as a reference standard. An animal was regarded as MC-PCR positive once a positive MC-PCR result was obtained in at least one of the tissues examined, brain, heart, breast muscle, thigh or drumstick. Of 67 inoculated chickens, 46 were MC-PCR positive in at least one sample.

In serum, IgG tests showed either substantial agreement (MAT, Kappa 0.70; ELISA, Kappa 0.67) or moderate agreement in case of IFAT (Kappa 0.58) with the results in MC-PCR on the individual chicken. YI was highest in MAT and lowest in IFAT (Table 5).

In tissue fluids, however, IFAT showed always at least substantial agreement with MC-PCR (Kappa values 0.69-0.82). In ELISA substantial agreement between MC-PCR and antibody detection was observed in meat juice from breast, thigh and drumstick muscle (Kappa 0.65-0.70) while there was only moderate agreement in case of meat juice from heart muscle (Kappa 0.54). MAT results showed relative to the MC-PCR results only in fluids collected from heart and drumstick musculature substantial agreement (Kappa 0.63) (Table 5).

In serum all tests showed 100% specificity in the non-inoculated birds. However, all tests showed deficiencies in specificity when applied to meat juices. In the non-inoculated chickens, MAT had a very low specificity of 25% once fluids collected from breast muscle were applied. A reduced specificity of MAT was also observed in non-inoculated chickens when fluids from heart, thigh or drumstick were applied (87.5%, 79.2%, 87.5%, respectively). In IFAT and ELISA a slightly reduced specificity was observed in heart fluids (IFAT, ELISA) or in tissue fluids collected from thigh musculature (ELISA) (Table 5).

The sensitivity of IFAT and ELISA was independent of the analyte generally 100% or slightly lower (IFAT, fluid collected from heart muscle). In MAT sensitivity did not reach 100% in tissue fluids (91.3-97.8) although the sensitivity of this tests applied to serum was 100%.

In tissue fluids IFAT showed higher YI than in serum (0.67-0.82). In ELISA (YI, 0.53-0.69) the opposite was the case, except for fluid from breast musculature. MAT used to test body fluids revealed the lowest YI (0.53-0.62) (Table 5).

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Table 5: Summary on the characteristics of serological tests relative to MC-PCR in chickens

Test Analyte	**Kappa**	**Kappa (95% CI)**	**Sensitivity**	**Specificity (all)**	**Specificity (non-inoculated)**	**Youden's index (all)**
MAT						
Serum	0.70	0.55-0.86	100.0% (46/46)	73.3% (33/45)	100% (24/24)	0.73
Heart	0.63	0.46-0.79	97.8% (44/46)	64.4% (29/45)	87.5% (21/24)	0.62
Breast	0.20	0.00-0.41	91.3% (42/46)	71.1% (32/45)	25.0% (6/24)	0.62
Thigh	0.58	0.41-0.75	95.7% (44/46)	57.8% (26/45)	79.2% (19/24)	0.53
Drumstick	0.63	0.47-0.79	91.3% (42/46)	71.1% (32/45)	87.5% (21/24)	0.62
Mean	*0.55*		*95.2%*	*67.5%*	*75.8%*	*0.62*
IFAT						
Serum	0.58	0.42-0.75	100.0% (46/46)	57.8% (26/45)	100% (24/24)	0.58
Heart	0.71	0.56-0.85	95.7% (44/45)	71.1% (32/44)	95.8% (23/24)	0.67
Breast	0.82	0.71-0.94	100.0% (46/46)	82.2% (37/45)	100% (24/24)	0.82
Thigh	0.74	0.60-0.88	100.0% (46/46)	73.3% (33/45)	100% (24/24)	0.73
Drumstick	0.69	0.54-0.84	100.0% (46/46)	68.9% (31/45)	100% (24/24)	0.69
Mean	*0.71*		*99.1%*	*70.7%*	*99.2%*	*0.70*
ELISA (Tg-SAG1)						
Serum	0.67	0.52-0.82	100.0% (46/46)	66.7% (30/35)	100% (24/24)	0.67
Heart	0.54	0.36-0.71	100.0% (46/46)	53.3% (24/45)	91.7% (22/24)	0.53
Breast	0.69	0.54-0.84	100.0% (46/46)	68.9% (31/45)	100% (24/24)	0.69
Thigh	0.70	0.55-0.86	100.0% (46/46)	57.8% (26/45)	95.8% (23/24)	0.58
Drumstick	0.65	0.49-0.80	100.0% (46/46)	64.4% (29/45)	100% (24/24)	0.64
Mean	*0.65*		*100.0%*	*62.2%*	*97.5%*	*0.62*

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4.2.3.2. Results in experimentally infected turkey

Serological response after experimental infection in turkeys

Turkeys were experimentally infected with tissue cysts (orally, one infected mouse brain), oocysts (10^3-10^6) or tachyzoites (intravenously, 10^6). Turkeys were slaughtered either 4, 8, 12 or 16 weeks p.i. (in case of one group of tachyzoite-infected turkeys) (Table 2). In case of turkeys infected by oocysts or tissue cysts animals were slaughtered 8 weeks p.i.

The serological response upon infection was determined using three serological tests, MAT, IFAT, TgSAG1 ELISA.

Table 6: Sensitivity and specificity of serological tests to detect *T. gondii* infection in experimentally inoculated turkeys

Serological test	Specificity	Sensitivity			
		Tissue cyst	Oocyst	Tachyzoite	Total
MAT	100% (18/18)	100% (18/18)	100% (40/40)	79.2% (19/24)	93.9% (77/82)
IFAT	100% (18/18)	100% (18/18)	100% (40/40)	62.5% (15/24)	89.0% (73/82)
ELISA (TgSAG1)	100% (18/18)	100% (18/18)	100% (40/40)	100% (24/24)	100% (82/82)

All tests showed a 100% specificity in non-inoculated control animals. The sensitivity of detecting experimentally inoculated turkeys varied between 79.2% (tachyzoites infection) and 100 % (oocyst infection). The highest total sensitivity was observed in ELISA (100%), followed by MAT (93.9%). The IFAT showed a lower sensitivity than ELISA and MAT (89%) Table 6.

In all tests the inoculation of tachyzoites – i.e. an infectious stage not relevant in nature – revealed the lowest proportion of seropositive turkeys ranging from 62.5% in IFAT to 100% in ELISA.

No effect on test sensitivity by infectious dose was observed.

Detectability of T. gondii by MC-PCR after experimental infection in turkeys

MC-PCR was applied to five different tissues (brain, heart, breast muscle, thigh, and drumstick) collected from the experimentally inoculated animals 4 to 16 weeks post inoculation.

The amount of tissue used for the different organs varied according to the size of the organ. Although only a mean of 1.29 g brain were used for "sequence capture" this organ tested positive in 66.7% of the inoculated turkeys. In case of heart 19.0 g and in case of breast muscle, thigh and drumstick 100 g of musculature were lysed and a proportion of this lysate corresponding to 4 g tissue was used for "sequence capture". In heart 61.0% and in breast, thigh and drumstick muscle only 34.1%, 48.1% or 54.9% of the turkeys tested positive by MC-PCR, respectively. Of all inoculated turkeys, 72.0% revealed a MC-PCR positive result, independent of the type of tissue analysed (Table 7).

The MC-PCR was 100% specific when applied to tissues from non-inoculated controls (i.e. all samples tested negative).

The sensitivity of the MC-PCR was clearly dependent from the type of stage used to inoculate the turkeys. While MC-PCR detected parasite DNA only in 2/24 turkeys inoculated by tachyzoites, those animals inoculated by oocysts or tissue cysts revealed a positive result in almost all turkey (97.5 - 100%).

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Independent of the type of inoculum the tissues ranked as follows with respect to the detectability of *T. gondii* by MC-PCR (reflection of anatomical distribution): brain > heart > drumstick > thigh > breast muscle (Table 7).

The number of parasites in the analyte was estimated using standard concentrations. Differences between heart or brain versus all remaining tissues were statistically significant (Dunn's method; P< 0.05). There was also a statistical significant difference in the estimated parasite concentrations between heart and brain. In case of the comparison between breast, thigh and drumstick no statistically significant difference was observed (Fig. 2).

Table 7: Sensitivity and specificity of MC-PCR to detect *T. gondii* DNA in tissues of experimentally inoculated turkeys

MC-PCR	Specificity	Sensitivity			
		Tissue cyst	**Oocyst**	**Tachyzoite**	**Total**
Brain	100% (15/15)	94.4% (17/18)	90.0% (36/40)	4.3% (1/23)	66.7% (54/81)
Heart	100% (18/18)	83.3% (15/18)	85.0% (34/40)	4.2% (1/24)	61.0% (50/82)
Breast	100% (17/17)	22.2% (4/18)	60.0% (24/40)	0.0% (0/24)	34.1% (28/82)
Thigh	100% (18/18)	47.1% (8/17)	77.5% (31/40)	0.0% (0/12)	48.1% (39/81)
Drumstick	100% (18/18)	61.1% (11/18)	85.0% (34/40)	0.0% (0/12)	54.9% (45/82)
All tissues	100% (18/18)	100% (18/18)	97.5% (39/40)	8.3% (2/24)	72.0% (59/82)

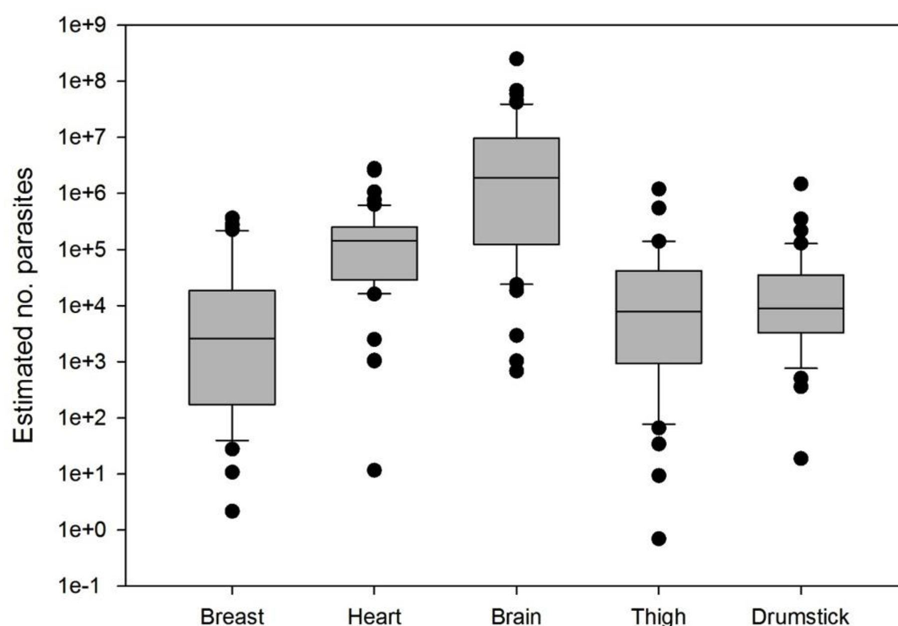

Figure 2: MC-PCR results on experimentally *T. gondii*-inoculated turkeys. Estimated number of parasitic stages in samples analysed. Differences between heart and brain as well as heart or brain versus all remaining tissues were statistically significant (Dunn's method; P< 0.05).

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Diagnostic characteristics of serological tests to identify MC-PCR T. gondii positive turkeys after experimental infection using serum or body fluids

For the examination of diagnostic characteristics of different antibody detection techniques applied to serum and various tissue fluids (heart, breast muscle, thigh, drumstick) the results of MC-PCR were used as a reference standard. An animal was regarded as MC-PCR positive once a positive MC-PCR result was obtained in at least one of the tissues examined, i.e. brain, heart, breast muscle, thigh or drumstick. Of 82 inoculated turkeys, 59 were MC-PCR positive in at least one sample.

IFAT revealed in serum and in all meat juices Kappa values between 0.65 and 0.76 suggesting a substantial agreement between the result of MC-PCR on individual turkey and antibody detection. The analysis by ELISA showed in all analytes (serum, meat juices) always Kappa values between 0.44 and 0.55 suggesting a moderate agreement. In MAT lower Kappa values were often observed. While MAT analysis of serum revealed a moderate agreement with the MC-PCR result, the MAT results obtained for meat juices revealed only a poor or fair agreement with the results observed by MC-PCR on turkey (Table 8).

Specificity in non-inoculated controls was optimal in all tests once serum was analysed. In MAT specificity was low (22.2-67.7%) when meat juices were analysed. ELISA also showed a slightly reduced specificity (93.8-94.4%) in meat juices from heart breast and thigh musculature. In IFAT the analysis of non-inoculated turkeys revealed a slightly reduced specificity in case of meat juice from drumstick musculature (94.4%) (Table 8).

In body fluids IFAT showed higher YI than in serum (0.70-0.73). In ELISA (YI, 0.40-0.48) the opposite was the case, except for fluid from heart musculature. MAT used to test body fluids revealed the lowest YI (0.11-0.31) (Table 8).

Table 8: Summary on the characteristics of serological tests relative to MC-PCR in turkeys

Test Analyte	**Kappa**	**Kappa (95% CI)**	**Sensitivity**	**Specificity (all)**	**Specificity (non-inoculated)**	**Youden's index**
MAT						
Serum	0.56	0.38-0.73	98.3% (58/59)	53.7% (22/41)	100% (18/18)	0.52
Heart	0.37	0.14-0.60	100% (59/59)	31.3% (10/32)	33.3% (5/18)	0.31
Breast	0.12	0.00-0.35	98.3% (58/59)	12.2% (5/41)	22.2% (4/18)	0.11
Thigh	0.30	0.09-0.51	100% (59/59)	26.8% (11/41)	55.6% (10/18)	0.27
Drumstick	0.31	0.10- 0.52	98.3% (58/59)	92.3% (12/41)	67.7% (12/18)	0.28
Mean	*0.33*		*99.0%*	*43.3%*	*55.8%*	*0.30*
IFAT						
Serum	0.65	0.49-0.81	98.3% (58/59)	63.4% (26/41)	100% (18/18)	0.62
Heart	0.73	0.61-0.86	100% (59/59)	67.7% (21/31)	100% (16/16)	0.68
Breast	0.72	0.58- 0.86	96.6% (57/59)	73.2% (30/41)	100% (18/18)	0.70

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Test Analyte	**Kappa**	**Kappa (95% CI)**	**Sensitivity**	**Specificity (all)**	**Specificity (non-inoculated)**	**Youden's index**
Thigh	0.76	0.63-0.89	98.3% (58/59)	75.0% (30/40)	100%) (17/17)	0.73
Drumstick	0.74	0.60-0.88	100% (59/59)	70.7% (29/41)	94.4% (17/18)	0.71
Mean	*0.72*		*98.6%*	*70.0%*	*98.9%*	*0.69*
ELISA (TgSAG1)						
Serum	0.48	0.29-0.67	100.0% (59/59)	43.9% (18/41)	100% (18/18)	0.44
Heart	0.55	0.35-0.75	100.0% (59/59)	48.4% (15/31)	93.8% (15/16)	0.48
Breast	0.46	0.27-0.65	100.0% (59/59)	41.5% (17/41)	94.4% (17/18)	0.41
Thigh	0.44	0.25-0.64	100.0% (59/59)	40.0% (16/40)	94.1% (16/17)	0.40
Drumstick	0.48	0.29- 0.67	100.0% (59/59)	43.9% (18/41)	100% (18/18)	0.44
Mean	*0.48*		*100.0%*	*43.5%*	*96.5%*	*0.43*

4.2.4. Discussion and conclusion

Anatomical distribution of T. gondii in tissues of chickens and turkeys

One of the aims of the EFSA project GP/EFSA/BIOHAZ/2013/01 was to generate information on the anatomical distribution of *T. gondii* in the tissues of infected chickens and turkeys. Tissues from chickens and turkeys, experimentally inoculated either orally (i.e. similar as possible in nature by tissue cysts or oocysts) or intravenously (experimental route; tachyzoites), were made available from a previous project for the EFSA project. In the present study, these tissues were examined by MC-PCR to define differences in the distribution of the parasite in different tissues of these infected birds. Due to the fact that these tissues had been stored frozen the analysis by mouse bioassay was no longer possible. However, previous experience and also the results of bioassay and MC-PCR on tissues of infected field chickens suggested that there is a good correlation between the results of mouse bioassay and the results of MC-PCR.

Results of the present study clearly indicate that in the experimentally infected birds (chickens, turkeys) the highest number of positive findings was obtained in the brain and the heart of about 2/3 of the experimentally inoculated animals. In the remaining tissues examined only about 1/3 up to 1/2 of the animals revealed a positive result in MC-PCR. The observation that in turkeys more than 1/2 of the animals tested positive in drumstick while in chickens only 1/3 of the animals rendered a MC-PCR positive result in the same tissue, could suggest that there are species-specific differences in the distribution of the parasite in tissues.

In addition, in turkeys, MC-PCR revealed statistically significantly higher parasite concentrations in brain tissue than in heart tissues. In contrast, in chickens, no statistically significant differences were observed between these tissues although brain and heart also tested as predilection site in this animal species. Parasite concentrations in samples from breast, thigh and drumstick musculature were usually about 100- to 1000-times lower than in brain or heart tissue in both animal species.

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In both experimental studies, the infection using tachyzoites induced seroconversion in many animals (more than 60%). However, in most of the birds (i.e. in all chickens and in more than 90% of the turkeys) no parasite DNA was detected at the end of the experiment. Results from prior investigations in 16 different organs of the same animals revealed positive results by conventional nested PCR in 33.3% of the chickens (4/12 infected animals) and 83.3% of the turkeys (20/24 infected animals) (Geuthner et al., 2014). The low extent of tissue positivity by MC-PCR indicates that infection led to a very low parasite density in turkeys. It may be questioned if the major part of the chickens developed detectable tissue stages at all. This could suggest that tissue cyst was impaired in the parasites directly retrieved from cell culture in contrast to parasites administered after animal passage (as tissue cysts or as oocysts).

Due to the observation that that birds inoculated by tachyzoites were often not found DNA positive we concluded that the situation in these birds may not reflect the natural situation due to the non-natural inoculation route (i.e., intravenous) and the tachyzoites stage most likely not causing infection in chicken under natural conditions. However, we still included these birds in further analysis, because they were inoculated with viable parasites and had seroconverted.

Relationship between seropositivity in chickens and presence of T. gondii in meat

As serological tests we used the modified agglutination test (MAT), a well-established in-house immunofluorescence test (IFAT) and an ELISA based on TgSAG1 (p30-ELISA (Maksimov et al., 2011)) as proposed by the European Food Safety Authority for the analysis of swine (EFSA, 2011).

In experimentally infected animals the results showed that sensitivity was usually very high, with the highest values by IFAT and ELISA (i.e. 100% or close to 100%) and slightly lower values in the MAT. Especially by MAT lower sensitivity values were observed when body fluids were analysed instead of serum. Consequently, when MAT is applied to body fluid instead of serum this test seems to be less sensitive compared to IFAT or ELISA. Compared to IFAT the ELISA also had a lower specificity in detecting MC-PCR positive chickens and turkey. However, in general the ELISA performed better than MAT.

In MAT a high number of body fluids from non-inoculated chickens and especially turkeys revealed a false-positive result. This low specificity at the cut-off applied in MAT to achieve enough sensitivity in body fluids was one of the reasons of an often low agreement between MAT and the MC-PCR results.

Conclusions on experimental infection study in chickens and turkey

1. Experimental inoculations using brains from chronically infected mice (tissue cysts) or oocysts were highly successful in inducing a MC-PCR detectable infection in chickens and turkeys. Tachyzoite inoculations were markedly less successful and rarely detectable by MC-PCR.
2. The results of samples positive by MC-PCR suggested that there is at least 100-fold more parasite DNA in brain or heart than in breast, thigh or drumstick musculature.
3. Under experimental conditions the agreement between MC-PCR results suggest moderate to substantial agreement between the IFAT and TgSAG1-ELISA results, independently of the analyte (serum, body fluids).
4. MAT shows a high proportion of false-positive reactions in body fluids, including fluids from tissues from non-inoculated controls. These false-positive reactions were responsible for a low agreement with MC-PCR results when body fluids from breast, thigh or drumstick were analyzed.
5. MAT results showed in serum and heart fluid similar agreements with the MC-PCR results as IFAT or TgSAG1-ELISA.

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5. General Conclusions and Recommendations

5.1. Conclusions

The objectives of the experimental studies described in this report were to:

(i) assess the relationship between indirect detection methods and direct detection methods for presence and levels of infective cysts in meat and other edible tissues.

(ii) evaluate the anatomical distribution of cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals.

(iii) collect information to identify on-farm risk factors for *T. gondii* infection in each animal species.

The experimental studies on *T. gondii* in meat were divided into four species-specific sections (ruminants, pigs, horses, poultry). The emphasis on different objectives and approaches varied by species, depending on existing knowledge gaps.

The species-specific results have been discussed in the corresponding chapters. In this section, the results are discussed by objective, thus providing a comparison between species and with the results from the extensive literature review.

5.1.1. Relationship between indirect and direct detection of *T. gondii*

A summary of the information on direct detection in seropositive and seronegative animals of the main livestock species collected in the extensive literature review is presented in Table 1. This overview shows a lack of information especially for turkeys (no entries), horses (3 entries from 2 records), cattle (4 entries from 3 records), and goats (4 entries from 4 records). Based on the literature review there was a better concordance between direct and indirect detection in pigs, small ruminants, and chickens than in cattle and horses.

The relationship between indirect and direct detection of *T. gondii* has been studied in cattle (section 1.1), in pigs (section 2.1), in horses (section 3) and in organic laying hens (section 4.1) and the results are summarized in Table 2. For cattle and horses, these studies were proposed due to the lack of data identified in literature. For pigs, no lack of data was identified in the extensive literature review. However, since samples were readily available from a national survey and only additional testing by MC-PCR was needed, this species was also included. For chickens, a lot of data is available from literature; however, in most of these studies, there was a difference between the direct detection method applied to seropositive animals (individual testing by mouse bioassay) and the method applied to seronegative animals (pooled testing by cat bioassay). This has led to a wide range in the estimate for the probability of direct detection in seronegative chickens, hence a study specifically designed to investigate the relationship between indirect and direct detection of *T. gondii* in chickens was proposed. In addition, available data in chickens are mainly on MAT and there are few studies comparing results in bioassay with those in other serological tests.

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Table 1: Overall, minimum and maximum percentage of direct detection of *T. gondii* (by cat bioassay, mouse bioassay or PCR) in seropositive and seronegative animals and concordance between direct and indirect (serological) detection (kappa-value) with interpretation by livestock species

Species	Detection in seropositives		Detection in seronegatives		Kappa-value[3] (95% CI)	Interpretation	Entries
	Overall[1] (n; 95% CI)	Range[2]	Overall[1] (n; 95% CI)	Range[2]			
Pigs	58.8% (592; 54.8-62.8%)	8-100%	4.9% (650; 3.3-6.6%)	0-75%	0.547 (0.495-0.599)	moderate concordance	17
Cattle	3.6% (111; 0.14-7.1%)	0-10%	2.4% (457; 1.0-3.8%)	2-3%	0.018 (<0-0.067)	no to poor concordance	4
Sheep	39.4% (1002; 36.4-42.5%)	5-100%	1.8% (922; 0.98-2.7%)	0-4%	0.366 (0.331-0.402)	fair concordance	17
Goats	34.9% (152; 27.3-42.4%)	0-72%	2.0% (50; 0.00-5.9%)	0-2%	0.198 (0.113-0.284)	poor to fair concordance	4
Chickens	53.4% (1679; 51.0%-55.8%)	0-100%	1.8%-17.4% (2153; 1.3-19.0%)	0-25%	0.370-0.543 (0.339-0.571)	fair to moderate concordance	76
Horses	8.8%-13.8% (80; 2.6-21.3%)	8-9%	2.4%-32.0% (540; 1.1-36.0%)	3%	<0-0.162 (<0-0.226)	no to poor concordance	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.

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For a comparison between indirect and direct detection of *T. gondii*, a reasonable number of infected animals is needed. For chickens this was achieved by focusing on organic laying hens and backyard reared hens, as their older age compared to broilers and the outdoor access implies that these chickens have a relatively high probability of exposure to *T. gondii* in combination with selection based on serological results. For pigs, this was also done by selecting animals for the mouse bioassay based on their serological results. For cattle and horses, no parameters that can easily be used for the selection at the slaughterhouse were identified in the literature review of risk factors. Also, as the data available from the literature review suggest a lack of concordance between the detection of antibodies and the presence of the parasite in cattle and horses, selection based on serological screening was not considered useful. Instead, the total number of animals tested by mouse bioassay in these studies was higher, and samples were collected in multiple countries from animals of different ages. None of the studies was designed to provide a reliable estimate of the prevalence of *T. gondii* infection, and especially when selective testing was applied (pigs and chickens), the sampled population was not representative for these livestock species. The results have been presented in the preceding sections, but are summarized for comparison to the results from the literature review in Table 2.

Table 2: Prevalence of direct detection of *T. gondii* in seropositive and seronegative animals by livestock species based on own experimental data

Species	Method	Detection in seropositive animals (total number examined)	Detection in seronegative animals (total number examined)
Cattle[1]	Mouse bioassay	3.3% (n=60)	1.2% (n=325)
	qPCR digest	0.0% (n=60)	0.0% (n=341)
	MC-PCR	5.1% (n=59)	4.3% (n=92)
Pigs[2]	Mouse bioassay	69.5% (n=46)	6.0% (n=83)
	qPCR digest	71.1% (n=52)	23.9% (n=88)
	MC-PCR	30.8% (n=52)	10.2% (n=88)
Horses[3]	Mouse bioassay	8.1% (n=98)	2.2% (n=178)
	qPCR digest	0.0% (n=50)	0.8% (n=123)
	MC-PCR	8.0% (n=100)	6.6% (n=182)
Organic laying hens[4]	Mouse bioassay	85.7% (n=28)	6.1% (n=33)
	qPCR digest	96.4% (n=28)	9.1% (n=33)
	MC-PCR	96.4% (n=28)	6.1% (n=33)

[1]Cattle were considered seropositive with a positive MAT at ≥1:6 serum dilution
[2]Pigs were considered seropositive with a positive MAT at ≥1:6 cardiac fluid dilution
[3]Horses were considered seropositive with a positive MAT at ≥1:6 serum dilution
[4]Organic laying hens were considered seropositive if at least two out of three serological tests (ELISA, IFAT and MAT) gave a positive result.

From Table 2 it is clear that a reasonable ratio of samples from seropositive- and seronegative pigs, horses and organic laying hens was tested. For cattle, the number of MAT-negative animals tested largely exceeded that of MAT-positive animals. However, the total number of seropositive cattle tested by mouse bioassay is slightly higher than in the pig or chicken studies. Overall, the sampled populations are suitable to study the concordance between direct and indirect detection of *T. gondii*.

In this study, direct detection rates in seronegative- and seropositive cattle and horses confirm the poor concordance between direct and indirect detection that was found in literature. For pigs, the proportion of direct detection in seropositive animals is as expected when using mouse bioassay and qPCR on the digest (69.5% or 71.1% respectively), but low for MC-PCR (30.8%). In seronegative pigs, the direct detection rate by mouse bioassay (6.0%) is similar to the proportion previously reported and mentioned in the extensive literature review (4.9%). In contrast, direct detection rates using qPCR on heart digest and MC-PCR on diaphragm are considerably higher (23.9% and 10.2%). However, since PCR-based methods can also detect non-viable parasites, this does not indicate that the risk for

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consumers from seronegative pigs is higher than indicated by the results of the mouse bioassay. In our study, *T. gondii* could be detected in nearly all seropositive organic laying hens (85.7-96.4%), which is similar to the highest values from studies included in the extensive literature review. The overall detection rate in seronegative chickens from literature has a wide range (1.8-17.4%), as seronegative chickens were often tested pooled, with the consequence that the exact number of positive chickens remained unknown when a pool turned out positive. Our results in individually tested seronegative chickens (6.1% and 9.1%) suggest that the probability to detect *T. gondii* in seronegative hens is only slightly higher than the 5% expected discordance level defined in the literature review.

It is interesting to note that, out of the two species with a good concordance between indirect and direct detection, the proportion of positive mouse bioassays in seropositive chickens (85.7%) was higher than in seropositive pigs (69.5%). Although this may have been influenced by methodological differences in determining the serological status of animals and by differences in the mouse bioassay protocol (e.g. limiting the analyses of mouse brains to MAT positive mice in the pig study and the use of pepsin-digestion and immune-compromised mice in the chicken study), it could partly reflect the fact that the entire heart was tested in the case of chickens, whereas only one sample of 200g was taken for the pigs, due to the size of the heart in this species.

Animals were tested by different direct detection methods used on different tissues. In our opinion, demonstration of the presence of the parasite in any tissue shows that the animal may pose a risk for consumers. In particular, it can be assumed that the probability that *T. gondii* is present in a meat cut (even if the tested edible tissue was found negative) is higher when the predilection site was found positive than when all direct detection results were negative. In poultry, this assumed relationship between the presence of *T. gondii* at the predilection site and in edible tissues was confirmed in the farm and experimental infection studies.

PCR-based methods (qPCR on digest and MC-PCR) cannot discriminate between viable and non-viable parasites. Therefore, in contrast to the mouse bioassay, these methods do not allow to directly predict the infection risk for consumers. However, also in this case, it can be assumed that an animal positive in a PCR-based method is more likely to also carry viable parasites than an animal that was negative by PCR.

In summary:

1. MAT-based detection of antibodies should not be recommended as an indicator for the presence of viable *T. gondii* infections in cattle and horses. Therefore, other serological tests need to be evaluated. As long as no reliable serological test is available for these species, the use of direct detection methods is preferred.
2. In contrast, in pigs and poultry, the detection of antibodies appears to be useful to estimate the extent of viable *T. gondii* infections and to estimate the infection risk for consumers. However, viable *T. gondii* was also detected in <10% of seronegative pigs and chickens. Therefore, serological test results (i.e. using serological tests applied in our studies) cannot be used to declare the meat of individual pigs and chicken as *T. gondii*-free. Moreover, our results clearly suggest that the ability to identify animals harboring *T. gondii* using an indirect detection method is influenced by the assay and the matrix/analyte used for antibody detection.
3. No studies in small ruminants were performed to examine the concordance between direct and indirect detection within the presented project. Based on literature review, the concordance between direct and indirect detection of *T. gondii* in sheep and goats is expected to be similar to the concordance observed in pigs and poultry.

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5.1.2. Anatomical distribution of *T. gondii* tissue cysts

The anatomical distribution of tissue cysts in the different livestock species has first been reviewed and discussed in the report of the extensive literature review. Based on the conclusion from the extensive literature review, tissues were selected for the slaughterhouse studies in cattle (section 1.1), pigs (section 2.1), horses (section 3) and chickens (section 4), a predilection tissue and a representative of edible tissue was tested. The overview of selected tissues is copied below (Table 3) as a summary of the results from the extensive literature review. Note that, although two types of tissues were tested in the slaughterhouse studies, different methods were used for predilection (mouse bioassay) and edible (MC-PCR) tissues in the case of cattle, pigs and horses. For that reason these results cannot be used to compare the parasite load in these tissues. In the field study on laying hens, mouse bioassay and MC-PCR were performed on both types of tissues and these results are included in the discussion below.

Table 3: Proposed sampling sites to represent a predilection site and edible tissue

Species	Predilection tissue	Edible tissue representative
Bovines (WP5)	Liver	Diaphragm
Pigs (WP6)	Heart	Diaphragm
Horses (WP7)	Heart	Diaphragm *
Chicken (WP8)	Heart	Drumstick/lower leg muscle**

* Diaphragm was chosen based on results in other animals, and because it was included in the pool of tissues tested in the publication by (Al-Khalidi and Dubey, 1979). However, no support for this tissue could be found in the overview.

** For chickens, the decision was based on the results from the systematic review data and additional data that were obtained through experimental infection.

In the experimental phase of the project, experimental infection studies were performed in calves (section 1.2), sheep (section 1.3), pigs (section 2.2) and chickens and turkey (section 4.2) to gain more insight into the anatomical distribution of tissue cysts. However, due to time constraints, these studies ran simultaneously with the slaughterhouse studies, therefore the selection of tissues was based solely on the extensive literature review. Here, we summarize and compare the results from the different experimental infection studies and discuss whether the results would have altered our selection of tissues for the slaughterhouse study.

Comparable experimental infection studies were performed at the Moredun Research Institute in calves, pigs and sheep. The results indicate that maximum differences in parasite load were less than 10-fold in calves and pigs, and approximately 50-fold for sheep. Thus, true predilection sites were not identified among the tissues tested. It is important to note that, for sheep and pigs, only skeletal muscle tissues have been tested by MC-PCR and variation in parasite load for these tissues may truly be limited. In the meantime, additional MC-PCR results have also become available in literature for goats, sheep and pigs. The results on individual tissues of experimentally infected goats demonstrate statistically significant differences in parasite load in liver (increased), heart (decreased) and dorsal muscle (increased) when comparing goats 30 and 90 days post infection, and indicate that the lung may represent a predilection site in this species (Jurankova et al., 2013). In sheep, brain was identified as the most frequently affected organ (6/6 experimentally infected animals) with highest parasite loads (<1-30,914 parasites/g), followed by lung (3/6 animals with <1-36.3 parasites/g) and heart (3/6 animals with <1 parasite/g). Few skeletal muscle samples tested positive and liver and spleen were negative in all animals (Jurankova et al., 2015). In six experimentally infected pigs, the highest concentration of *T. gondii* DNA was found in brain tissues, equivalent to [median] 553.7 (range 3857.7-121.9) parasites per gram, followed by lungs, heart and dorsal muscles with median values corresponding to 0.3 (range 61.3-0.02); 2.6 (range 7.34-0.37) and 0.6 (range 2.81-0.31) parasites per

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gram of tissue, respectively. Skeletal muscles from fore and hindlimb, liver and kidney presented very low infection burdens equivalent to [median] ≤0.2 parasites per gram of tissues, and no parasite DNA could be detected in the spleen (Jurankova et al., 2014). The lack of variation in parasite load between the various skeletal tissues in these pig and sheep studies parallels the results obtained in our pig and sheep studies. In our calf study, even though several tissues other than skeletal muscle (including brain) have been tested, no clear predilection sites could be identified.

In the experimental infection studies in poultry (section 4.2 and (Koethe et al., 2015)) performed at Leipzig University predilection sites were clearly identified. In chicken, brain and heart were more frequently affected than breast, thigh or drumstick. The parasite loads in positive tissues were similar in brains and hearts, but significantly higher (100-1000 fold) than in positive breast, thigh or drumstick muscle. The results in turkey were similar, although in this species brain had a higher parasite load than heart, which in turn had a higher parasite load than breast, thigh or drumstick. In the slaughterhouse study in chicken (section 4.1), heart and drumstick were tested by mouse bioassay and MC-PCR, and these results confirm that also in naturally infected chickens, heart is more likely to be positive and has higher parasite loads than drumstick.

From comparing our calf, sheep and pig experimental infection studies, it is clear that sheep show much higher parasite loads in tissues than calves (Cq-values in MC-PCR ranging between 22.64-28.23 compared to 32.34-35.54), even though the inoculation dose for calves (10^6) was similar to the dose used for sheep (5 x 10^5). Pigs had parasite loads similar to the calves (Cq-values between 33.61 and 35.65), but they were inoculated with a much smaller dose (10^3 tissue cysts or oocysts). Pooling of tissues differed by species (5g of 1 or 2 sheep, 10g of 5 pigs, and 33.3g of 2 or 3 calves) and may have affected these results; nevertheless, a low susceptibility and a difficulty to recover parasites from experimentally infected calves corresponds with what has been described in literature (Dubey, 1986).

In pigs, chickens, and turkeys, oocyst and tissue cyst inoculation were used. Oocysts contain 8 infective sporozoites and tissue cysts can contain a range of infective bradyzoites. For that reason, differences in inoculation-stage are likely associated with differences in inoculation dose. The pigs that were inoculated with 10^3 oocysts or 10^3 tissue cysts did not show any clear differences. In chickens and turkeys, inoculated with a specified number of oocysts or one infected mouse brain, a higher recovery rate was observed in the oocyst-inoculated chickens, but the ranking of tissues (reflecting the anatomical distribution) was not affected in either of the species.

As the calf experimental infection study did not give a clear indication of predilection sites, it would not have influenced our selection of tissues for the slaughterhouse study. When designing the slaughterhouse study, the use of either liver or heart as a predilection site has been discussed extensively, with the final choice of liver being based on the literature review as results from these experimental infections were not yet available. Diaphragm was chosen as a representative of edible tissue. Retrospectively, we have selected suitable tissues, but possibly not the optimal tissue for mouse bioassay. The choice between liver and heart would not have been affected as liver and heart tested positive in mouse bioassay and MC-PCR with similar results in the pooled testing and neither of the two tissues was MC-PCR positive for calf 118, which turned out to be the most heavily infected calf after individual testing. However, heart and liver were not among the tissues that were most convincingly positive in mouse bioassay (i.e. tongue, brain, diaphragm, semitendinosus and psoas major muscle). Based on these results, we might have selected diaphragm for mouse-bioassay (as tongue is difficult for digestion, brain is not available for sampling in the slaughterhouse, and semitendinosus and psoas major are expensive meat cuts). In the current design, the diaphragm was tested by MC-PCR.

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The tissues selected for the slaughterhouse study in pigs (heart and diaphragm) have not been tested in the experimental infection study, and no experimental infection study was done for horses. The results from the experimental studies in chickens and poultry were already partly available when designing the slaughterhouse study, and have been taken into account for the selection of tissues.

In summary:

1. There is little variation in parasite load between different skeletal muscles in pigs and sheep. Clear predilection sites (brain, heart and lung) have been identified in MC-PCR based studies described in the literature.

2. *T. gondii* was detected in several types of tissues of experimentally infected calves, but no clear predilection sites were identified.

3. Brain and heart were identified as predilection sites in experimentally infected chickens and turkeys. Estimated parasite loads were 100 to 1000-fold higher than in breast, thigh or drumstick muscle of the same animals.

4. The comparison of recovery rates and parasite loads in the different species is hampered by differences in study design and sampling. However, the results appear to confirm the lower parasite load in cattle as suggested by the literature.

5. Oocyst and tissue cyst inoculation were performed in the pig, chicken and turkey infection experiments. There was no evidence that the inoculation route affected the anatomical distribution in any of the species.

6. Selection of tissues for the slaughterhouse studies:

a. Cattle: As no clear predilection sites were identified in the calf infection experiment, this would not have influenced the selection of tissues for the bovine slaughterhouse study. The selected tissues gave positive results in the experimental infection study.

b. Pigs: Heart and diaphragm have not been tested in our pig infection experiment, but results from literature confirm that heart is a predilection site and the best choice for sampling when the brain is not available. Diaphragm was selected as a representative of edible tissue based on the extensive literature review. It was not tested in our pig infection experiment or in the MC-PCR based study described in literature. However as both our study and the study published by others found little variation in the parasite load between different skeletal muscles, diaphragm is still considered a suitable representative of edible tissue.

c. Horses: Within this project, no experimental infection study has been performed in horses. The selection of tissues for the slaughterhouse study was based on limited data available in the literature; therefore the anatomical distribution of tissue cysts in horses remains an important gap in knowledge. At this point, it remains unknown whether clear predilection sites exist in horses.

d. Chickens: In the experimental infection study, brain and heart gave similar results and were both identified as predilection sites. There was limited variation in the parasite load in breast, thigh and drumstick. This corresponds nicely with literature and justifies our selection of tissues for the slaughterhouse study.

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5.1.3. On-farm risk factors for *T. gondii* infection

On-farm risk factors have been discussed as part of our extensive literature review. The diagram showing the putative routes of on-farm transmission is shown below (Fig. 1).

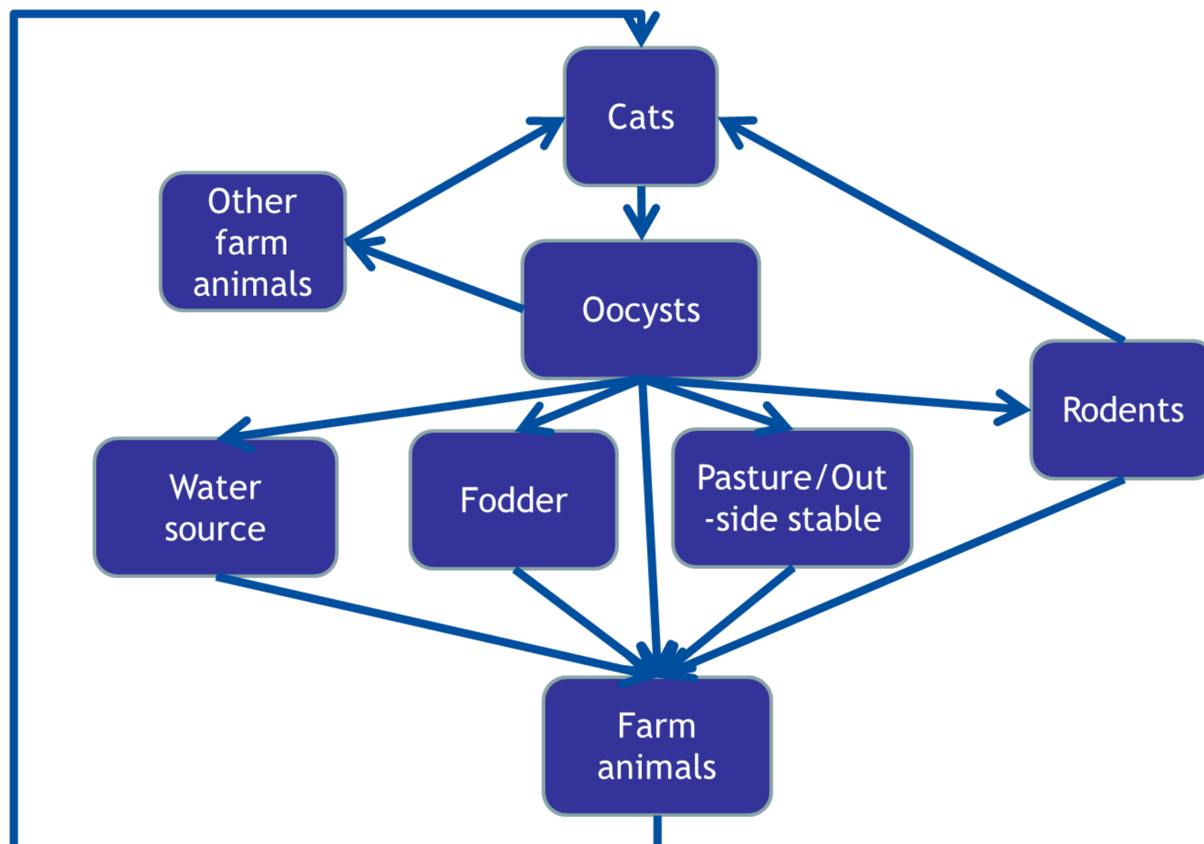

Figure 1: Putative routes of on-farm transmission of *T. gondii*

An overview of risk factors categorised by the following categories was presented:

- Definitive host-related variables
- Variables characterizing the level of confinement of animals and management intensity
- Variables characterizing the likelihood of fodder contamination
- Variables characterizing the likelihood of water contamination
- Rodent-related variables

Most of the studies described in the literature focussed on pigs or small ruminants. There was a lack of studies on chicken, cattle and horses.

In the experimental phase of our project, specific risk factor studies were performed for indoor-housed dairy goats in The Netherlands (section 1.4) and pigs in the UK (section 2.3), and the slaughterhouse

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study in chickens was additionally used to investigate potential risk factors (section 4.1). In addition, vaccination as a protective factor was studied in vaccination and challenge experiments in sheep (section 1.3) and pigs (section 2.2).

Although studies were already available in literature on small ruminants, the effect of confined housing was not very clear for ruminants: in most species confined housing protects but for sheep and cattle the opposite was observed. In the Netherlands, dairy goat farming has intensified in the past decades. This provides a unique situation to study the effects of confined-housing in a ruminant species, and therefore a study in indoor-housed dairy goats in the Netherlands was designed. Although risk factor studies in pigs were reported in literature, no information on the risk factors for *T. gondii* infection in pigs in the UK was available. Therefore, the Food Standards Agency and the Royal Veterinary College planned to perform a study. These data have also been made available for this EFSA project. The field study in chickens was designed differently from the other slaughterhouse studies, and has allowed us to also investigate the presence of potential on-farm risk factors in chickens, an important knowledge gap identified in the literature review.

Exposure to *T. gondii* varies more importantly at the farm-level than at the level of the individual animal. Therefore, farms are the study unit in these epidemiological studies, and a farm-level classification of the presence of *T. gondii* is associated with the presence of potential risk factors. In all three risk-factor studies it was difficult to include a large number of farms (goats n=52, pigs n=73, chickens n=19), which has negatively affected the power to find statistically significant associations. Especially in the pig study, the power was further reduced because only on 5 out of the 73 farms were deemed positive.

The seroprevalence in indoor housed dairy goats in the Netherlands appears low in comparison to sheep in the Netherlands and in comparison to Dutch goats studied in the nineties, as well as in comparison to goats in other countries, thus suggesting that indoor housing provides some protection. Nonetheless, 13.3% of goats tested positive for anti-*T. gondii* IgG and 61.5% of 52 farms had at least one seropositive goat present. Presence of cats and the mean age of the animals were found independent predictors of *T. gondii* seroprevalence at farm level by multivariable modelling.

In the pig study, the seroprevalence was low (3.6%) and most of the infected pigs came from a small number of farms (6.8%). The low number of positive farms has resulted in a lack of power, and no statistically significant associations were identified in univariate analysis of putative risk factors and a ≥50% probability that pigs with antibodies to *T. gondii* are present at the farm. Observed trends were in line with risk factors described in literature (e.g. presence of cats, open feed storage).

The chicken study was limited to laying hens with outdoor access from small (≤50) and large farms (≥500 chickens). Risk factors have only been studied in univariate analysis. Several variables related to the presence of cats were identified as risk factors. None of the large farms had a within herd prevalence of *T. gondii* infection ≥20%, even when cats were present. Many variables were associated with large farm size and identified as putative protective factors (high chicken density, presence of empty period, long cleaning interval and large premises), but it remains unclear whether these factors are independent predictors of exposure to *T. gondii*.

A live-attenuated vaccine to prevent *T. gondii* abortion in sheep is commercially available. Within Europe, the vaccine is used in few countries including the UK and France. In vaccinated ewes, dissemination of the parasite to the placenta and foetus during a challenge infection was very limited (Buxton et al., 1991). Therefore, a reduction of tissue cyst formation could also be expected. This reduction and (nearly) complete prevention of tissue cyst formation has now been shown for sheep and pigs vaccinated using the S48 strain *T. gondii* (sections 1.3 and 2.2 (Burrells et al., 2015; Katzer et al.,

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2014)). Vaccination thus is a potential preventive measure that can reduce the risk for consumers. However, since the antibody response in vaccinated can not be differentiated from the response in naturally infected sheep, vaccination can interfere with the correlation between the detection of antibodies and the presence of *T. gondii* in tissues. Therefore serological screening in sheep cannot be used to investigate the risk for consumers when vaccination is systematically applied and individual vaccination status is not known.

In the cattle and horse slaughterhouse studies, information on the animals was collected from the slaughterhouse with the intention to study the association between these data and the presence of *T. gondii*. In both cattle and horses, a lack of concordance between the detection of antibodies and presence of *T. gondii* was demonstrated. Potential risk factors should therefore be evaluated for an association with the results based on a direct detection method rather than an indirect detection method. As only 13 cattle tested positive by a direct detection method, the data were not suitable for statistical analysis. Instead a table with a description of the positive animals has been included in the report. In horses, similar results were found, with only 12 horses tested positive by mouse bioassay, and therefore data were not suitable for statistical analyses.

In summary:

1. Indoor-housed dairy goats in the Netherlands are exposed to *T. gondii*, but the seroprevalence appears to be relatively low. The presence of cats was associated with increased on-farm seroprevalence.

2. The exposure to *T. gondii* in pigs in the UK appears to be low and infected pigs are present at a small number of farms.

3. For laying hens with outdoor access in Germany, the risk of *T. gondii* infection was higher in backyard systems or at small farms compared to large organic farms.

4. Experimentally, vaccination with S48-strain *T. gondii* has been shown to reduce or prevent tissue cyst development in sheep and pigs.

5.2. Recommendations

Considering the three topics for these experimental studies the following recommendations have been formulated:

(i) the relationship between indirect detection methods and direct detection methods for presence and levels of infective cysts in meat and other edible tissues

The literature review and the slaughterhouse studies indicate that, in cattle and horses, indirect detection (by applying MAT as a serological tool in the slaughterhouse studies) does not correlate well with the detection of the parasite using direct methods (mouse bioassay, qPCR on digest and MC-PCR). Therefore, in these species, direct detection methods are preferred to obtain an indication of the risk for consumers. For this reason, implementation of serological screening to identify high risk herds or animals at farms or slaughterhouse level is not recommended for cattle and horses, using serological methods currently available. The development of new methods that provide an indication of the presence of parasites is considered a research priority, as current direct detection methods are not feasible for large-scale testing.

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In pigs, chickens and small ruminants there was a fair to substantial concordance between the presence of antibodies to *T. gondii* and the presence of the parasite in tissues. For these species, serological testing can be used to identify high risk herds or animals at the farm or slaughterhouse. However, since viable parasites were also detected in <10% of seronegative pigs and organic laying hens, a negative result in an indirect test cannot be used to declare the meat of that animal as *T. gondii* free. In addition, since the sensitivity of direct detection methods is limited due to the fact that only a fraction of the total body volume of the animal was tested, direct detection applied to small samples probably may have underestimated the fraction of seropositive animals that harbored *T. gondii*.

Vaccination will give rise to an antibody response and has been shown to reduce tissue cyst formation in sheep and pigs. Therefore, a lack of agreement between serological results and presence of tissue cysts is expected for vaccinated animals. Toxovax is available in many European countries, but except for the UK and France it is hardly used. Vaccination status should be considered if serological testing would be implemented to identify high risk sheep flocks or sheep.

(ii) the anatomical distribution of the cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals

Considering the results from the extensive literature review and from the experimental infection study carried out in calves, it appears that cattle can become infected with *T. gondii* but clear predilection sites for *T. gondii* dissemination were not identified for this species. Further experimental studies for the purpose of studying the anatomical distribution in cattle are not advised, unless more sensitive detection methods become available. The hypothesis that cattle can clear *T. gondii* tissue cysts needs to be studied experimentally.

Very few studies were available to assess the anatomical distribution of *T. gondii* in horses and no data were added in the experimental phase of this project. Therefore, the anatomical distribution of *T. gondii* in horses remains an important gap in knowledge. Experimental infection in horses is needed to study anatomical distribution and identify preferred tissues for testing. It is possible that, similarly to cattle, no real predilection sites can be identified in horses. However, if clear predilection sites are identified, and these are found to be different from the heart and diaphragm (selected for the slaughterhouse study), the concordance between direct and indirect detection needs to be reassessed with direct detection applied on the predilection tissues.

In pigs, small ruminants and poultry true predilection sites (e.g. brain and heart) were identified in literature and, for poultry, also in the experimental studies. In general, tissue cysts were less likely to be detected in skeletal muscle or meat cuts, and when parasites were detected in skeletal muscle the parasite load was generally low compared to predilection sites. Also, in the experimental infection in sheep and pigs there appeared to be little variation between the different skeletal tissues. In the extensive literature review, the results for different muscle and meat samples were also combined into one category. This combined category generally ranked high and similarly to the predilection sites. For that reason, an animal that is found positive in a predilection site can be assumed to also carry tissue cysts in skeletal muscle, and should be considered a potential risk for consumers. Applying direct detection methods on a predilection site will make most efficient use of resources to determine the prevalence of animals harbouring tissue cysts.

(iii) on-farm risk factors for *T. gondii* infection in livestock.

The extensive literature review identified a lack of risk factor studies for cattle, horses and poultry. In cattle and horses, a negative serological result did not exclude exposure and the lack of direct detection in seropositives raised doubt about the specificity of the measured antibody response. For

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that reason, epidemiological studies aiming to identify risk factors for infection in cattle and horses should preferably be based on the results obtained with a direct rather than on results with current indirect detection methods.

Data from our studies demonstrated that indoor farming of goats does not prevent exposure to *T. gondii*. Therefore, in the case of goats, housing information is unlikely to be useful for risk classification in the slaughterhouse. The study in laying hens (on organic farms and backyard farms) showed that outdoor access alone was not sufficient to explain the extent by which hens were infected. Other risk factors may represent additional traits necessary to characterize the on-farm risk of infection.

In an experimental setting, it has been shown that vaccination reduces tissue cyst formation in sheep and pigs. The value of vaccination as a potential intervention measure should be further evaluated by mathematical modelling and, if positive, a field study.

The extensive literature review of risk factors was mostly based on cross-sectional studies. Intervention studies are needed to determine the effectiveness of preventing exposure to risk factors that have been identified in cross-sectional studies.

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6. Appendices

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6.1. Appendix A: Protocols cattle, pigs and horses

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6.1.1. Protocol of the mouse bioassay

Protocol for bioassay in mice following a tissue (organ) digestion
(According to the test used in previous national surveys - ovine, bovine, pigs- in France)

6.1.1.1. Protocol for tissue digestion

1. Determine sample weight
2. Clean the sample from all fat and connective tissues
3. Cut the sample in small pieces (approx. 1cm^3).
4. Ground the small pieces in the grinder or briefly in the blender until the consistency of tartar staek (hamburger). (If the tissue is ground too much you'll end up with a larger digest resulting in a smaller fraction of the total volume to be inoculated in the mice)
5. Weigh 200g of sample and transfer it to a conical erlenmeyer with a screwing cap.
6. Prepare the digestion solution. Mix for this purpose trypsin, physiological saline (0.9% NaCl) and antibiotics (penicilline-streptomycine, amoxyciline) for each sample as proportioned for 200g:
   - *30ml – trypsin (Sigma Aldrich, Trypsin from porcine pancreas ref: T4674, bottles of 100ml) http://www.sigmaaldrich.com/catalog/product/sigma/t4674*
   - *270ml – physiological saline (0.9% NaCl)*
   - *6ml – Penicilline-streptomycine (for example: Fisher Scientific Penicillin-Streptomycine in solution ref: 11548876, bottles of 100 ml)*
   - *0.6ml – Amoxyciline (for example: GlaxoSmithKline, powder of 1gr + solvent , 5ml injectable; ref:K128501)*

   (In case there is less than 200g of sample available: take note of the sample weight and make the proportion to obtain the right amount of digestion solution.)

7. Mix in erlenmeyer (1l, screw cap) the ground sample with 306.6ml digestion solution
8. Incubate the sample rocking for 1.5 hours at 37°C (e.g. on a rocking platform set at on 200 RPM).
9. Prepare another 2 erlenmeyers (0.5l), funnel and gauze (Girodmedical, nonwoven compresses 40gr OPTIMA LCH 10cm x 10cm, ref:CNT400) for filtration.
10. Put the funnel on the erlenmeyer and put the double layered gauze inside the funnel in order to make gauze filter.
11. Pour down digested sample and mix it gently while all liquids pass through the gauze filter.
12. Take the ends of the gaze filter and squeeze the sample to obtain additional liquids from the sample.
13. Divide the gained liquid into 4-6 conical tubes of 50ml.
14. Centrifuge at +4°C at 1800 x g for 10 minutes with the use of centrifuge brake (max.rate: 9).
15. Discard supernatant and keep only the pellet.
16. Dilute pellet in 15ml physiological salt (0.9%) in the same tube and vortex it until the pellet is completely resuspended and combine resuspended pellets in one or two tubes.
17. Centrifuge at +4°C at 1800 x g for 10 minutes with the use of centrifuge brake (max. rate: 9).
18. Discard supernatant and keep only the pellet.
19. Dilute pellet in 15ml physiological salt (0.9%) in the same tube and vortex it until the pellet is completely resuspended. If still in two tubes, combine in the pellets in one tube.
20. Centrifuge at +4°C at 1800 x g for 10 minutes with the use of centrifuge brake (max. rate:9).
21. Discard supernatant and take out 1ml of the pellet/digest to be stored at -20°C for PCR (using a cut pipet tip).

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22. Resuspend remainder of the digest in 500ul of each antibiotic solution (see II, protocol for mouse bioassay) and make up to 5ml total volume with physiological saline. Aliquot 2x 1ml in eppendorf tubes for the mouse bioassay and store it at +4°C overnight until inoculation in mice (to allow antibiotics to work), or, with same day (fresh) samples you can inoculate directly.
23. Store the remainder of the digest at 4°C for a maximum of three days, and use it to reinoculate a mouse if mortality occurs. After three days transfer the remainder to storage at -20°C.

Additional instructions:

- Trypsin and peni-strepto are packed in 100ml plastic bottles. Once opened they can be stored in refrigerator on +4°C for 2 weeks (15 days).

6.1.1.2. Protocol for mouse bioassay

REAGENTS

- 1 ml of tissue digest
- Antibiotics: streptomycin, peni-strepto, amoxycilin, ciprofloxacin, cefotaxime, vancomicin.
- Physiological saline (0.9% NaCl)
- 2 Swiss OF1=Swiss Webster/CD1 female mice minimum 6-8 weeks old

PROCEDURE

- Prepare two antibiotic solutions in the following concentrations:

Solution 1:

- 6 ml of physiological saline (0.9% NaCl)
- 4 ml Peni-strepto – 100 µl/1ml of inoculums = 10000 U/ml;
- 0.4 ml Amoxycilin – 100 µl/1ml of inoculums = 1g/5ml;

Solution 2:

- 4 ml of physiological saline (0.9% NaCl)
- 50 µl Ciprofloxacin –concentration of 2 mg/ml
- 15 µl Cefotaxime –concentration of 100mg/ml;
- 20 µl Vancomicin –concentration of 2mg/ml

- In each cage separate 2 mice and mark the cages
- In one ml syringe prepare the digest with the antibiotics (see point 22 above)
- Grip the mouse by the skin from the neck and back and immobilize it in the hand so that their abdomens are exposed for manipulation.
- Sterilize mouse abdomen with 70% alcohol
- On the right side from the Linea Alba, approximately in the second third of the abdomen, inoculate the tissue digest into the abdominal cavity of the mouse.
- Monitor mice according to institute regulations.
- Optional: Take blood sample for serology at 4 weeks to identify positive mice for strain isolation at cull
- At 6 weeks post-inoculation take a blood sample for serology and take the mouse brain for direct detection of parasite by PCR (additional microscopy is optional)

In case mice are found dead:

- Within 3 days after inoculation: bacterial contamination of digest is most likely. Mouse does not need to be sampled. Use the remaining digest to inoculate a new mouse.
- Between 3 and 14 days post inoculation: acute toxoplasmosis is likely; collect peritoneal fluid for PCR (and optional microscopy)

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- At least 14 days post inoculation: tissue cysts may be detectable at this point; collect brain for PCR (and optional microscopy).

In case mice have to be culled early (humane endpoint):
- As above, but also collect blood for serology from 14 days post inoculation onwards.

Humane endpoints:
Depends on institutional guidelines but, for your reference, the scoring system used at Moredun is provided.

Animal Scoring System:

Category	Description	Score
A	Sleek/glossy coat	0
Coat Condition	Ruffled coat	1
(max score 2)	Stary stiff coat	2
B	Bright and active	0
Demeanour	Hunched	1
Scoring cumulative	A reluctance to move	1
(max score 3)	Tottering gait	1

Total Score = A + B
Animals will be killed using a Schedule 1 procedure if an animal has reached a maximum score in either category (A or B) or if an animal has a total score of 3 for three consecutive days.

6.1.1.3. Protocol for detection of infection in mice

A mouse bioassay was considered positive if at least one mouse was positive in serology or PCR.

Serology:
Use serology as available in your laboratory. Store serum sample at -20°C to allow confirmation by MAT in Reims.

PCR on mouse brain: as described in Section 2

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Parasite isolation (optional):
Parasite isolation is optional but encouraged. You can try to keep the strain going by further mouse inoculation or tissue culture in your lab, but you can also send mouse brain or peritoneal exudate to Isabelle in Reims. In Reims brain or exudate will be frozen at the conditions that assure best conservation of isolates. Brains containing cysts will be stored in their Biological Resource Centre and they will send isolates if any partner ask these in future. If genotyping of isolates shows an interesting genotype (unusual), they can assume multiplication of parasites by cell cultures and store several aliquots of tachyzoites.

Which mice: if you have the opportunity to collect blood at 4 weeks and do serology this will help prepare for positive mice. If not, you may choose to send samples from mice with clinical signs of toxoplasmosis, or perform serology or PCR (microscopy) on the brain samples within a few days after culling to have the results available while the remainder of the brain is still relatively fresh (store at 4°C in physiological saline with penstrep/amoxy). Fresh brain and peritoneal exudate samples in physiological saline with penstrep/amoxy can be send to Reims at 4°C.
Alternatively, you can store a brain suspension or peritoneal exudate from all mice in liquid nitrogen until serology and PCR results are available, and send them to Reims frozen.

Storage in liquid nitrogen:
Add to the brain (after grinding) 10 mL of physiologic saline
Centrifugation at 2500 rpm/min for 10 min (at room temperature).
Remove the supernatant and add slowly 800 µL of solution of DMSO 20% (cooled at + 4°C) on the pellet of brain and then obtain homogeneous sample.
Put total of sample in a cryotube and homogenize it by carefully pipetting up and down.
Freeze the cryotube at + 4°C then -80°C in stages and not directly at -80°C.
After a stage of -80°C, we can store brain (in DMSO) in liquid nitrogen and sent to Laboratory of Parasitology (Reims) by a transporter.

Please notify Isabelle Villena or Dominique Aubert before sending material! (ivillena@chu-reims.fr and daubert@chu-reims.fr) Address is provided with MAT details.

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6.1.2. Protocols for PCR based techniques

6.1.2.1. Protocol for DNA isolation from tissue digest and mouse brain

Use Nucleospin Tissue from Macherey-Nagel (Cat. No. 740952).
http://www.mn-net.com/tabid/1353/default.aspx
Standard protocol for human or animal tissue and cultured cells.

For digests:

1. Use a 2 ml reaction tube
2. 200 µl digest; 1440 µl T1; 200 µl Proteinase K; digest 1-3 hours [additional T1 and Proteinase K has to be ordered]
3. Take out 230 µl of the final suspension into a fresh tube and add 200 µl B3
4. Proceed with M&N protocol as described (step 3)

For mouse brains:
Homogenise at least half of the brain in PBS (75ul PBS per 25mg of tissue) and use 100ul of this homogenate as described in M&N protocol.

Suggested homogenization procedure (other methods are accepted):
Each brain is collected in a sterile 5ml bijoux containing 1ml (check if this equals to 75ul per 25mg!) sterile PBS. The entire mouse brain is homogenised by passing through an increasing gradient of fine gauge needles, an 18G needle is attached to a 5ml plastic syringe and the mouse brain passed through the needle at least five times to form a homogenate. The brain homogenate is then passed through a 21G needle, ensuring that the brain material could pass through each needle five times.

6.1.2.2. Protocol for the sequence specific magnetic capture of DNA

MC-PCR on the edible tissues from cattle will be performed at RIVM, the Netherlands; and from horses and pigs at ENVA, France.

Preparation of crude DNA extract

A hundred gram meat sample (free of fat and connective tissue) was cut into pieces of approximately 1 cm^3. Knives and forceps were thoroughly rinsed with hot water and soap, and thereafter cleaned with DNAzap (Lifetechnologies, AM9890, 2x250ml €110) or new scalpel blades were used for every sample to prevent cross-contamination. The cut tissue was put into a Stomacher400 bag with filter (Seward, BH6041 STR 200 bags = €+- 137, BagPagePlus, 400 ml, Interscience, France), and cell lysis buffer containing 100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0, 0.2% SDS, 200 mM NaCl, 40 mg/l proteinase K (30 mAnson-U/mg; Merck 70663, Carl Roth GmbH, Karlsruhe, Germany) was added at 2.5 ml per gram of sample. The sample was homogenized in the Stomacher400 (Seward, Worthing, United Kingdom) for 2 min at high speed. The bag was sealed, and the sample was incubated overnight at 55 °C in a water bath. After incubation, the samples were homogenized for 1 additional minute. Afterwards, 50 ml of homogenate was transferred to a 50-ml tube, and centrifuged for 45 min at 3500×g. Twelve millilitres of supernatant (crude extract) was transferred to a 15-ml polypropylene tube.

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Removal of free biotin

Twelve millilitres of crude extract was incubated at 100°C for 10 min to inactivate the proteinase K. Per sample, 50 µl of streptavidin sepharose (binding capacity 300 nmol/ml; GE Healthcare, 17-5113-01; €393,00 5ml) was washed 3 times in phosphate buffered saline (PBS). After cooling down the crude-extract samples to below 40°C, the washed streptavidin-sepharose was added. The samples were incubated for 45 min at room temperature, while rotating at 10 rpm, to allow for streptavidin–biotin binding. Afterwards, the tubes were centrifuged for 15 min at 3500×g, and 10 ml of biotin-free supernatant was transferred to a clean 15-ml polypropylene tube.

Sequence-specific magnetic capture

Ten picomoles of Tox-CapF and Tox-CapR were added to each supernatant. The supernatants were heated at 95°C for 15 min to denature all DNA. Then, the tubes were transferred to a shaking water bath set at 55°C, and left to allow for hybridization between capture oligonucleotides and *T. gondii* DNA for 45 min. The tubes were left to cool down to room temperature while rotating at 10 rpm for 15 min. Per sample, 80 µl of M-270 Streptavidin Dynabeads (Invitrogen, 65306; €1336,00 10ml) or hyBeads Streptavidin (Hyglos, Bernried, Germany) was washed in Binding & Washing (B&W) buffer (5 mM Tris HCl pH 7.5,0.5 mM EDTA pH 8.0, 1 M NaCl) according to the manufacturer's instructions. The washed beads and 2 ml of 5 M NaCl were added to each supernatant sample and the samples were incubated rotating (10 rpm) at room temperature for 60 min. The complex of streptavidin bead and biotin-labelled capture-oligonucleotide with hybridized *T. gondii* DNA was isolated using the DynalMPC-1magnet or DynaMag-15 magnet (Invitrogen 12001D €282 or Promega PolyATract System 1000 Cat.No. Z5410 €65).The tube was placed in the magnet for 10min and supernatant was removed with a disposable Pasteur pipette. The beads were washed twice in B&W buffer, and resuspended in 50 µl of distilled water in a 1.5-ml tube. The bead suspension was heated at 100°C for 10 min to release *T. gondii* DNA from the beads. The tube was placed in the Dynal MPC-S magnet (Invitrogen A13346, €409) or DynaMag-2 magnet (Invitrogen), and the supernatant was immediately transferred to a clean 1.5-ml tube. Beads were discarded.

6.1.2.3. Protocol for quantitative PCR

Quantitative PCR is to be used for magnetic capture samples as well as DNA isolated from digest and mouse brains. For samples from sequence-based magnetic capture you'll need to use the 529bp repeat region with primers annealing within the region captured using the capture oligos.
Within a study design the same qPCR has to be used for all samples, but there can be variation between species/study designs according to institute preferences (e.g. thermocycler, mastermix, probe-labelling). However, we will all use an aliquot of the same stock of control DNA for quantification purposes (provided by RIVM).
The RIVM-protocol is detailed below <u>as an example</u>.

For a PCR reaction to be considered positive all negative or blank controls in the PCR reaction have to be negative, the Cq-value has to be <40 and the shape of the amplification curve has to be similar to the positive controls. If so, samples with a Cq-value <35 are considered positive, samples with a Cq-value between 35 and 40 are additionally confirmed by identification of the correct band (162bp) in gel electrophoresis.
Real-time quantitative PCR on 529-bp repeat element (RE)
PCR amplification was performed in 96-wells plates using a LightCycler480 thermal-cycler instrument (Roche, Almere, the Netherlands). The 20 µl reaction mixture consisted of 4 µl 5× concentrated
Taqman master mix (Roche), 0.7 µM of each primer (Tox-9F and Tox-11R) (Table 1), 0.1 µM of Tox-TP1, 0.2 µM of CIAC-probe, 0.02 fg of CIAC, and 10 µl of template DNA. The reaction mixture was initially incubated at 95°C for 10 min to activate FastStart DNA polymerase. Initial incubation was

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followed by 45 amplification cycles that consisted of a denaturation step at 95 °C for 1 s, an annealing step at 58°C for 20 s, and an extension step at 72°C for 20 s. Afterwards, the samples were cooled to 40°C for 5 s. The temperature transition rate was 4.40°C/s for an increase in temperature and 2.20°C/s for a decrease in temperature. Fluorescence at 530 nm (Tox-TP1) and 560 nm (CIAC-probe) was measured at the end of each extension step. A *T. gondii* DNA standard series was included on each run for calculation of the standard curve, and estimation of PCR efficiency (>1.85) and error (<0.05). For each sample, the fluorescence-by-cycle-curve was used to calculate the fractional cycle number or crossing point (Cq) at which the second derivative was at its maximum (LightCycler software, Roche). All samples with a Cq-value that show a smooth exponential-amplification curve were scored positive, all samples without a Cq-value but with a positive CIAC-PCR were scored negative. Samples without a Cq-value and with a negative CIAC-PCR were retested.

Table 1: Primers, probes and capture-oligos used for *T. gondii* MC-PCR

Name	Sequence 5'-3'	5'label	3'label	Position[a]
Tox-CapF	cttggagcca cagaagggac agaagtcgaa ggggactaca gacgcgatgc cgctcctcca gccgtcttgg	Biotin TEG	-[b]	173–242
Tox-CapR	aagcctccga ctctgtctcc ctcgccctct tctccactct tcaattctct ccgccatcac cacgaggaaa	Biotin TEG	-	406–475
GRA6-CapF	gatttgtgtt tccgagcagg tgacctgggt cgctttttg aaacagcagg aaaacagctt cgtggtgcca cgtagcgtgc ttgttggcga ctacc	Biotin TEG	-	301–395
GRA6-CapR	gcagttcgta cagattccta cgttcttctc cacgtcggac agtgccgctc tcccggtcgt tgcatggcat cgactacaag acatagagtg cc	Biotin TEG	-	1145–1236
Tox-9F	aggagagata tcaggactgt ag	-	-	243–264
Tox-11R	gcgtcgtctc gtctagatcg	-	-	386–405
Tox-TP1	ccggcttggc tgcttttcct	6-FAM	BHQ1	338–357
CIAC-F	aggagagata tcaggactgt agccagcccg catcact	-	-	NA[c]
CIAC-R	gcgtcgtctc gtctagatcg atctgtaaag ttaacagatg tgctagt	-	-	NA
CIAC-	agcgtaccaa caagtaattc tgtatcgatg	JOE	BHQ1	NA

[a] position on GenBank AF146527 for Tox-oligonucleotides, and on GenBank L33814 for GRA6-oligonucleotides; [b] - : no label; [c] NA: not applicable

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PCR mix using 2x concentrated LC 480 Probes Master (Roche)
http://lifescience.roche.com/shop/en/us/products/lightcycler14301-480-probes-master
Ref. 04707494001 5 x 1 ml for up to 500 reactions of 20 µl final volume $345.00

No samples	**45**						
	concentration		**ul**	**x**	**samples**	**=**	**Total (ul)**
	stock	**final**					
Primer/probe mix	20X	1X	1,00	x	45	=	45,00
LC Probes Master	2X	1X	10,00	x	45	=	450,00
CIAC	0,0178fg/ul		1,00	x	45	=	45,00
Total without template			**12,00**				540,00
Template			8,00				

500ul 20X primer/probe mix			concentration in mix
100pmol/ul	Tox 9F	70ul	14pmol/ul
100pmol/ul	Tox 11R	70ul	14pmol/ul
100pmol/ul	Tox-TP-1	10ul	2pmol/ul
100pmol/ul	CIAC-probe	20ul	4pmol/ul
	qPCR water	330ul	

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6.1.3. Protocol for the MAT

MAT is performed in Reims.
To send sera:

- Aliquot 200ul-1ml (100ul is absolute minimum) in 1.5ml tube labelled with animal ID. Use safe-lock tubes or wrap in parafilm before sending.
- Store sera at -20° or 4°C until sample collection is complete, send all sera in one batch to:

E F S A
LABORATOIRE DE PARASITOLOGIE
Pr. I. VILLENA
HOPITAL MAISON BLANCHE
45, RUE COGNACQ-JAY
51092 REIMS CEDEX
FRANCE
TEL : (00) (33) 3 26 78 42 20
FAX : (00) (33) 3 26 78 73 28

- Inform Isabelle and Dominique by email of the expected delivery date (ivillena@chu-reims.fr and daubert@chu-reims.fr)

MODIFIED AGGLUTINATION TEST (According to the test used in previous national surveys - ovine, bovine, pigs- in France)

- Put at room temperature the reagents to be used
  * PBS pH7.2 (BIOMERIEUX, France)
  * BABS (BIOMERIEUX, France)
  * Figurative antigen (produced by the lab of Reims)
  * DTT 1M stock solution (7.725g DTT+ 0.04g sodium acetate QS 50mL MilliQ water then filtered with 0.45µm filter and frozen under 1mL)

- At use, prepare 1/17 dilution of figurative antigen in BABS buffer
- Sera dilution 1/3 in PBS pH 7.2 (25µL of serum + 50µL PBS)
- Preparation of DTT (stock solution) dilution 1/50 in PBS pH7.2

PROCEDURE
- Fill 25µL of serum diluted (1/3 prepared previously) in the first well of the plate
- Distribute 25µL of DTT diluted (1/50 prepared previously) across the line of the plate
- Make a ½ serial dilution of the sera to be tested, across the line
- Distribute 25µL of diluted antigen (1/17 prepared previously) across the line
- use a rocking platform set at on 200 RPM for 1 minute to homogenize the content of the plate
- Cover the plate with an adhesive film
- Incubated overnight at room temperature
- Reading the next day: a positive result is considered when the agglutination is more than 50% of the well
- Results will be expressed in dilutions: first well=6; second well=12
- If you want to express the titer, must be T = dil. / 2, 4, 8 depending on the batch of antigen

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6.2. Appendix B: Interpretation of kappa-values

Kappa	Interpretation
< 0.00	no concordance
0.00 – 0.20	poor (none to slight)
0.21- 0.40	fair
0.41 – 0.60	moderate
0.61 – 0.80	substantial
0.81-1.00	almost perfect concordance

Based on: Cohen J. A coefficient of agreement for nominal scales. Educ Psychol Meas. 1960; 20: 37–46.

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6.3. Appendix C: Questionnaire indoor-housed Dutch dairy goat farms

English-translated version of questionnaire provided to the goat farmers.

1. Are or were (stray) cats present at the farm in the past two years?
   □ Yes □ No (skip to question 5)

2. What was the maximum number of cats present at any given time?
   □ <5 □ 5-10 □ >10

3. Do the cats access the stable(s)?
   □ Yes □ No □ Don't know

4. Were there young cats (kittens) present at the farm within the past two years?
   □ Yes □ No □ Don't know

5. In the past 2 years, have you had problems with mice or rats at the farm?
   □ No □ Yes, namely ……

6. What water supply do you use for the goats' drinking water?
   □ Tap water □ Ground water (pump) □ Other, namely …..

7. Do you feed with an automated mixer-feeder?
   □ Yes □ No

8. What type of silage do you use? (Tick all applicable options)

	Lambs	Milking goats	Replacement stock
Straw	□	□	□
Grass hay	□	□	□
Grass silage	□	□	□
Corn silage	□	□	□
Lucerne (fresh)	□	□	□
Lucerne hay	□	□	□
Fresh grass	□	□	□
Other: …	□	□	□

9. In case silage is used, what type of silage storage do you use:
   □ Horizontal silo (bunker) □ Bales

10. Did (some of) the milking goats ever have outdoor access at your farm or a different farm?
    □ No □ Don't know □ Yes: (specify) …..

11. Did you follow closed-farm management in the past two years?
    □ Yes, closed for males and females
    □ Yes, completely closed for females, but males were introduced
    □ No, both females and males were introduced

12. Is other livestock present at the farm? If yes, please specify the species.
    □ No □ Yes: (specify) …..

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6.4. Appendix D: Questionnaire pig farms in the United Kingdom

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:___________________________

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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:* ________________________

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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

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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:* ______________________________

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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 clean between batches?**
    □Yes, it is always cleaned between batches
    □Yes, most of the times 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 disinfect between batches?**
    □Yes, it is always disinfected between batches
    □Yes, most of the times is disinfected between batches
    □Rarely

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

15. **Are staff designated to work exclusively 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

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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

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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!

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6.5. Appendix E: Protocols Poultry

6.5.1. Poultry 1. Mouse-Bioassay employed in studies on chickens (FLI protocol)

Reagents

- PBS (pH 7.2)
- Acid-Pepsin-Solution:
Pepsin, 2.6 g (1:10000 biological activity, Sigma Chemical, St. Luis, MO, USA),
NaCl 5.0 g dissolved in dd H2O, 500ml,
add HCl 25%, 7.0ml, adjust pH 1.10-1.20
- Sodium bicarbonate 1.2% (pH~8.3)
- RPMI medium with 1000 units penicillin and 100 µg of streptomycin per ml.

Procedure

1. Cut tissues with scissors into small pieces. Remove fat and connective tissues.
2. Grind tissue pieces with pistil and mortar or homogenize with a hand blender.
3. Place ground tissue in 50 ml conical tubes.
4. Incubate homogenate at 37°C (5 min).
5. Add 25 ml/5g of freshly prepared acid pepsin solution (pre-warmed at 37°C) and incubate at 37°C on a shaker for 60 min.
6. Filter the homogenate through two layers of gauze and centrifuge 25 ml of the filtered homogenate in a 50 ml conical tube at 1200 x g for 10 min.
7. Pour off the supernatant. Suspend the sediment in 2 ml PBS (per 5 g of tissue digested). Neutralize the homogenate with 1.5 ml of freshly prepared 1.2% sodium bicarbonate (pH 8.3) per 5 g of tissue initially digested. Centrifuge at 1200 x g for 10 min.
8. Pour off the supernatant and add 1500 µl of cell culture medium (per 5 g of tissue initially digested), that contains 100 units penicillin and 100 µg of streptomycin per ml.
9. Inoculate 0.5 ml of tissue homogenate subcutaneously into each of n=2 gamma-interferon-knockout mice (GKO mice) or n=2 gamma-interferon-receptor-knockout mice (GRKO mice) per sample.
10. In mice dying or developing toxoplasmosis (ruffled hair; apathy) after inoculation, infection is confirmed by PCR using DNA extracted from brain of inoculated mice using the protocol, essentiall as decribed (Talabani, H., Asseraf, M., Yera, H., Delair, E., Ancelle, T., Thulliez, P., Brezin, A.P., Dupouy-Camet, J., 2009, Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol 47, 2131-2135).
11. Mice are followed for 42 days. Sera of all mice surviving infection until 30 days post inoculation are examined by TgSAG1-ELISA.

6.5.2. Poultry 2. Magnetic-Capture protocol employed in studies on chickens (modified protocol based on the protocol of Opsteegh et al., 2010).

Preparation of crude DNA extract

Up to hundred gram meat sample (free of fat and connective tissue) was cut into pieces of approximately 1 cm^3. New, sterile, single-use scalpels were used for every sample to prevent cross-contamination. The cut tissue was put into a stomacher bag with filter (BagPagePlus, 400 ml, Interscience, France), and cell lysis buffer containing 100 mM Tris HCl pH 8.0, 5 mM EDTA pH 8.0,

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0.2% SDS, 200 mM NaCl, 40 mg/l proteinase K (30 mAnson-U/mg; Carl Roth GmbH, Karlsruhe, Germany) was added at 2.5 ml per gram of sample. The sample was homogenized in the stomacher bag (Interscience, France) for 2 min at high speed. The bag was sealed, and the sample was incubated overnight at 55 °C in a water bath. After incubation, the samples were homogenized for 1 additional minute. Afterwards, 50 ml of homogenate was transferred to a 50-ml tube, and centrifuged for 45 min at 3500×g. Twelve ml of supernatant (crude extract) was transferred to a 15-ml polypropylene tube.

Removal of free biotin

Twelve ml of crude extract was incubated at 100 °C for 10 min to inactivate the proteinase K. Per sample, 50 µl of streptavidin sepharose (binding capacity 300 nmol/ml; GE Healthcare, VWR, Germany) was washed 3 times in phosphate buffered saline (PBS). After cooling down the crude-extract samples to below 40 °C, the washed streptavidin sepharose was added. The samples were incubated for 45 min at room temperature, while rotating at 10 rpm, to allow for streptavidin–biotin binding. Afterwards, the tubes were centrifuged for 15 min at 3500×g, and 10 ml of biotin-free supernatant was transferred to a clean 15-ml polypropylene tube.

Sequence-specific magnetic capture

Ten picomoles of Tox-CapF and Tox-CapR were added to each supernatant. The supernatants were heated at 95 °C for 15 min to denature all DNA. Then, the tubes were transferred to a shaking water bath set at 55 °C, and left to allow for hybridization between capture oligonucleotides and *T. gondii* DNA for 45 min. The tubes were left to cool down to room temperature while rotating at 10 rpm for 15 min. Per sample, 80 µl of hyBeads Streptavidin Hyglos, Bernried, Germany) was washed in Binding & Washing (B&W) buffer (5 mM Tris HCl pH 7.5,0.5 mM EDTA pH 8.0, 1 M NaCl). The washed beads and 2 ml of 5 M NaCl were added to each supernatant sample and the samples were incubated rotating (10 rpm) at room temperature for 60 min. The complex of streptavidin bead and biotin-labelled capture-oligonucleotide with hybridized *T. gondii* DNA was isolated using the DynaMag-15 magnet (Invitrogen).The tube was placed in the magnet for 10 min and supernatant was removed by decanting. The beads were washed twice in B&W buffer, and resuspended in 50 µl of distilled water in a 1.5-ml tube. The bead suspension was heated at 100 °C for 10 min to release *T. gondii* DNA from the beads. The tube was placed in the DynaMag-2 magnet (Invitrogen), and the supernatant was immediately transferred to a clean 1.5-ml tube. Beads were discarded.

A positive (10^3 tachyzoites in 100 g breast muscle) and a negative (100 g breast muscle without tachyzoites) extraction control were included every time the procedure was performed.

Real-time quantitative PCR on 529-bp repeat element

PCR amplification was performed in 96-wells plates using the StepOnePlus thermal-cycler instrument (Life Technologies, Darmstadt, Germany). The 27.5 µl reaction mixture consisted of 1× concentrated reaction buffer, 5.5 mM $MgCl_2$, dNTP-Mix (0.2 mM dATP, dCTP, dGTP, 0.4 mM dUTP), 0.01 U/µl uracil-N-glycosylase, 0.025 U/µl TrueStart Hot Start DNA polymerase (Fermentas/Thermo Scientific, St. Leon-Rot, Germany), 0.9 µM of each primer (Tox-SC forward: 5'-GAGGGGGTGGCGTGGTT-3' and Tox-SC revers: 5'-GCGTCGTCTCGTCTRGAT-3') (Tox-9F and Tox-11R), 0.2 µM of Tox-TP, and 2.5 µl of template DNA. The reaction mixture was initially incubated at 50 °C for 10 min to allow uracil-N-glycosylase (UNG) to destroy any remaining uracil-containing DNA. This was followed by 10 min initial denaturation at 95 °C to inactivate UNG and to activate TrueStart DNA polymerase. This was followed by 45 amplification cycles that consisted of a denaturation step at 95 °C for 15 s, an annealing step at 50 °C for 30 s, and an extension step at 72 °C for 15 s. After completion of all cycles, the samples were cooled to 15 °C. The temperature ramp rate was set at 100 %. Fluorescence was measured at the each annealing step. A standard series of DNA from serially diluted tachyzoites (10^6

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to 10^2 per 100 g of meat) was included on each run for calculation of the standard curve, and estimation of PCR efficiency. For each sample, the quantity of *T. gondii* equivalents was calculated by comparing Cq-values of samples with the standards (StepOnePlus software, LifeTechnologies). All samples with a Cq-value that show a smooth exponential-amplification curve were scored positive, all samples without a Cq-value were scored negative.

Criteria for interpretation of real-time-PCR results

Real-time PCR results were expressed as Cq values. Results with Cq values < 35 were regarded as positive. Results with Cq values >40 were regarded as negative. In case of Cq 35-40 the respective amplification curves were visually inspected. In cases the curves diverged strongly from those of positive controls the samples were recorded as negative.

6.5.3. Poultry 3. TgSAG1-ELISA for chicken, turkey and mice:

Affinity purified 30 kDa *T. gondii* surface antigen (TgSAG1; Hosseininejad et al., 2008; Maksimov et al., 2011) was diluted in bicarbonate buffer (0.1 M, pH 8.3) and 120 µl/well of the diluted antigen (30 ng/ml) used to sensitize (1 h, 37°C) PolySorp ELISA plates (Nunc, ThermoFischer Scientific, Denmark). The plates were then washed three times with phosphate buffer saline (PBS) supplemented with 0.05 % (v/v) Tween® 20 (Serva, Heidelberg Germany) (PBST). A blocking step with 1% Casein in PBST (CasPBST; 30 min, 37°C) followed. The plate was emptied and 100 µl of each serum sample, diluted in CasPBST, was added (30 min, 37°C). Serum had been diluted 1:200 CasPBST while body fluids were diluted 1:20 in CasPBST. After sample incubation plates were washed three times with PBS-T. Species-specific conjugates (rabbit anti-chicken IgY-POD; rabbit anti-mouse IgG (H+L)-POD; A9046, Sigma, Deisenhofen, Germany) were diluted 1:4000 in CasPBST. 100 µl of diluted conjugate were added to each well and incubated (30 min, 37°C). Subsequently, the plates were washed three times with PBST and twice with distilled water. 1 % tetra-methyl-benzidine (TMB) with 0.012% (v/v) H_2O_2 was added to each well. After 15 min at 37°C, the reaction was stopped by addition of 50 µl 4 N H_2SO_4 and the optical density in each well read at 450 nm. Each sample was tested in duplicate. Positive (PC) and negative control (NC) experimental chicken sera (Maksimov et al., unpublished) or mouse sera (Schares et al., unpublished) were tested in quadruplicate on each plate. ELISA index values (I) were calculated for each sample (S) based the means of two OD values: IS=(ODS – ODNC)/(ODPC-ODNC).

6.5.4. Poultry 4. IFAT for chicken and turkey:

IFAT: Ten µl of a suspension of cell culture-derived *T. gondii* RH strain tachyzoites ($5x10^6$ ml^{-1}) in PBS were used to sensitize IFAT slide wells. Slides were air-dried and stored frozen at -20°C until used. The slides were fixed with ice-cold acetone for 10 min and then incubated in PBS for 10 min. Chicken sera were titrated in PBS in 2-fold steps starting at a serum dilution of 1:50 or a body fluid dilution of 1:5. The test was performed as described for *N. caninum* (Schares et al., 1998) but with the following modification: Anti-chicken IgG (H&L) produced in goat and coupled to FITC [Rockland Immunochemicals 603-102-002] diluted 1:50 in PBS, 0.2% Evans Blue was used to detect primary antibodies. The slides were examined using an Olympus IX50 or AHBT3 Axiovert fluorescence microscope (Olympus, Hamburg, Germany). Only complete peripheral fluorescence of the tachyzoite was considered specific. A titre of 50 was used as the positive cut-off titre in serum and a titre of 1:5 in body fluids.

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6.5.5. Poultry 5. Questionnaire on farms

Questionnaire for characterization of a laying hen farm (translation) Farm:

Name of the interviewee:

Function of the interviewee on farm:

Stocking number within the farm unit the sampled animals derived:

Other details on farm unit:

Date of interview:

1. Presence of cats

1.1. Farm premises

1.1.1. Cats are present on farm premises **(yes / no / do not know)**

1.1.2. No of cats per week on farm premises **(…..** [Number] **/ do not know)**

1.2. Chicken run

1.2.1. Cats have access to chicken run **(yes / no / do not know)**

1.2.2. No. of cats observed on chicken run per week **(…..** [Number] **/ do not know)**

1.3. Henhouse/hall

1.3.1. Cats have access to henhouse/hall **(yes / no / do not know)**

1.3.2. No. of cats per week in henhouse/hall **(….** [Number] **/ do not know)**

2. Level of confinement

2.1. Henhouse

2.1.1. Doors, windows, aeration are secured to prevent entrance of cats

(yes / no / do not know)

2.1.2. Doors, windows, aeration are secured to prevent entrance of rodents

(yes / no / do not know)

2.2. Chicken run

2.1.1. Run is secured that way that cats are not able to get in **(yes / no / do not know)**

2.1.2. Run is secured that way that rodents are not able to get in **(yes / no / do not know)**

3. Details on henhouse/hall

3.1. Kind of ground:……………………………

3.2. Kind of bedding:………………………………..

3.3. Size of henhouse/hall in sq m:…… ………………..

3.4. Hygienic measures in henhouse hall (yes / no)

3.4.1. Kind of hygienic measure

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3.4.1.1. Frequency of bedding change:………………………………

3.4.1.2. Frequency of cleaning and desinfection:……………………………

3.4.1.3. Which desinfectants is used?:……………………… ……………………

3.5. Empty period **(yes / no)**

3.5.1. Duration of empty period:………… …………………………

3. Details and size of chicken run

3.1. Kind of ground:……………… ……………………………………………..

3.2. Plant cover (yes / no / partially)

3.3. Size of chicken run in sq m:……………………..

3.4. Hygienic measure in chicken run (yes / no)

3.4.1. Kind of hygienic measure

3.4.1.1. Move to other run **(yes / no)**

3.4.1.2. Use of quick lime **(yes / no)**

3.4.1.3. Other measure:……………………………………………………………….

3.5 How long are the hens on a single chicken run: …………………..

4. Contamination of fodder

4.1. Storage

4.1.1. Storage protected from cats and cat faeces **(yes / no / do not know)**

4.1.2. Storage protected from rodents **(yes / no / do not know)**

4.1.3. A cleaning of silo is performed **(yes / no / do not know)**

5. Rodent control

5.1. A rodent control performed?

5.1.1. I poison used for rodent control? **(yes / no / do not know)**

5.1.2. Are cats used for rodent control? **(yes / no / do not know)**

5.1.3. Frequency of rodent control:…… ………………………..

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ABBREVIATIONS

ANSES –USC EpiToxo	French Agency for Food, Environmental and Occupational health and Safety
BA	Mouse Bioassay
DLO-CVI	Central Veterinary Institute
ELISA	Enzyme Linked Immunosorbent Assay
EFSA	European Food Safety Authority
ENVA –JRU BIPAR	National Veterinary School of Alfort
EU	European Union
FLI	Friedrich-Loeffler-Institut, Greifswald-Insel
FSA	Food Standards Agency
IFAT	Immunofluoresence Antibody Test
IMR	University of Belgrade Institute for Medical Research
ISS	Instituto Superiore di Sanità
MAT	Modified Agglutination Test
MC-PCR	Magnetic Capture Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
RIVM	National Institute for Public Health and the Environment
RVC	Royal Veterinary College
TgSAG1	*Toxoplasma gondii* SAG1 antigen
qPCR	Quantitative Polymerase Chain Reaction
UASVM CN	University of Agricultural Science and Veterinary Medicine, Cluj-Napoca

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REFERENCES

Al-Khalidi, N.W., Dubey, J.P., 1979. Prevalence of Toxoplasma gondii infection in horses. J Parasitol 65, 331-334.

Antonis, A.F.G., van Knapen, F., Dercksen, D.P., Jager, P.M., 1998. Toxoplasmosis in goats in the Netherlands: a pilot study. Tijdschr Diergeneeskd 123, 561-565.

Bartoń, K. 2014. MuMIn: Multi-Model Inference. http://CRAN.R-project.org/package=MuMIn.

Batz, M.B., Hoffmann, S., Morris, J.G., Jr., 2012. Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. J Food Prot 75, 1278-1291.

Beauvais, W., Orynbaev, M., Guitain, J., in preparation. Bayesian estimation of farm seroprevalence adjusting for multistage sampling and uncertainty in test performance – Brucella in southern Kazakhstan.

Benavides, J., Maley, S., Pang, Y., Palarea, J., Eaton, S., Katzer, F., Innes, E.A., Buxton, D., Chianini, F., 2011. Development of lesions and tissue distribution of parasite in lambs orally infected with sporulated oocysts of Toxoplasma gondii. Vet Parasitol 179, 209-215.

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

Boireau, P., Vallee, I., Roman, T., Perret, C., Mingyuan, L., Gamble, H.R., Gajadhar, A., 2000. Trichinella in horses: a low frequency infection with high human risk. Vet Parasitol 93, 309-320.

Bokken, G.C.A.M., Bergwerff, A.A., van Knapen, F., 2012. A novel bead-based assay to detect specific antibody responses against Toxoplasma gondii and Trichinella spiralis simultaneously in sera of experimentally infected swine. BMC Vet. Res. 8, doi: 10.1186/1746-6148-1188-1136.

Burrells, A., Benavides, J., Canton, G., Garcia, J.L., Bartley, P.M., Nath, M., Thomson, J., Chianini, F., Innes, E.A., Katzer, F., 2015. Vaccination of pigs with the S48 strain of Toxoplasma gondii--safer meat for human consumption. Vet Res 46, 47.

Buxton, D., Thomson, K., Maley, S., Wright, S., Bos, H.J., 1991. Vaccination of sheep with a live incomplete strain (S48) of Toxoplasma gondii and their immunity to challenge when pregnant. Vet Rec 129, 89-93.

Cavalcante, A.C.R., Carneiro, M., Gouveia, A.M.G., Pinheiro, R.R., Vitor R.W.A., 2008. Risk factors for infection by Toxoplasma gondii in herds of goats in Ceará, Brazil Arq. Bras. Med. Vet. Zootec. 60, 36-41.

CBS 2015. Agriculture; crops, livestock and land use by general farm type, region. (http://statline.cbs.nl/StatWeb/publication/).

Cheney, T., Powell, L.F., 2013. Prevalence of Salmonella, Toxoplasma, Yersinia, Hepatitis E and Porcine Reproductive and Respiratory Syndrome virus in UK pigs at slaughter. In: Safepork, Portland, USA, pp. 57-59.

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

de Moraes, E.P., da Costa, M.M., Dantas, A.F., da Silva, J.C., Mota, R.A., 2011. Toxoplasma gondii

The present document has been produced and adopted by the bodies identified above as author(s). In accordance with Article 36 of Regulation (EC) No 178/2002, this task has been carried out exclusively by the author(s) in the context of a grant agreement between the European Food Safety Authority and the author(s). The present document is published complying with the transparency principle to which the Authority is subject. It cannot be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

diagnosis in ovine aborted fetuses and stillborns in the State of Pernambuco, Brazil. Vet Parasitol 183, 152-155.

Djurkovic-Djakovic, O., Nikolic, A., Bobic, B., Klun, I., Aleksic, A., 2005. Stage conversion of Toxoplasma gondii RH parasites in mice by treatment with atovaquone and pyrrolidine dithiocarbamate. Microbes Infect 7, 49-54.

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary epidemiologic research. VER Inc; 2nd edition.

Dubey, J.P., 1986. A review of toxoplasmosis in cattle. Vet Parasitol 22, 177-202.

Dubey, J.P., 2009a. Toxoplasmosis in pigs-The last 20 years. Vet Parasitol 164, 89-103.

Dubey, J.P., 2009b. Toxoplasmosis in sheep--the last 20 years. Vet Parasitol 163, 1-14.

Dubey, J.P., Desmonts, G., McDonald, C., Walls, K.W., 1985. Serologic evaluation of cattle inoculated with Toxoplasma gondii: comparison of Sabin-Feldman dye test and other agglutination tests. Am J Vet Res 46, 1085-1088.

Dubey, J.P., Rajendran, C., Ferreira, L.R., Martins, J., Kwok, O.C., Hill, D.E., Villena, I., Zhou, H., Su, C., Jones, J.L., 2011. High prevalence and genotypes of Toxoplasma gondii isolated from goats, from a retail meat store, destined for human consumption in the USA. Int J Parasitol 41, 827-833.

Dubey, J.P., Thulliez, P., 1993. Persistence of tissue cysts in edible tissues of cattle fed Toxoplasma gondii oocysts. Am J Vet Res 54, 270-273.

Dubey, J.P., Verma, S.K., Ferreira, L.R., Oliveira, S., Cassinelli, A.B., Ying, Y., Kwok, O.C.H., Tuo, W., Chiesa, O.A., Jones, J.L., 2014. Detection and Survival of Toxoplasma gondii in Milk and Cheese from Experimentally Infected Goats. J. Food Protect. 77, 1747-1753.

EFSA, 2011. Technical specifications on harmonised epidemiological indicators for public health hazards to be covered by meat inspection of swine. EFSA Journal 9.

Esteban-Redondo, I., Maley, S.W., Thomson, K., Nicoll, S., Wright, S., Buxton, D., Innes, E.A., 1999. Detection of T. gondii in tissues of sheep and cattle following oral infection. Vet Parasitol 86, 155-171.

Gamble, H.R., Dubey, J.P., Lambillotte, D.N., 2005. Comparison of a commercial ELISA with the modified agglutination test for detection of Toxoplasma infection in the domestic pig. Vet Parasitol 128, 177-181.

Garcia-Bocanegra, I., Cabezon, O., Hernandez, E., Martinez-Cruz, M.S., Martinez-Moreno, A., Martinez-Moreno, J., 2013. Toxoplasma gondii in ruminant species (cattle, sheep, and goats) from southern Spain. Journal of Parasitology 99, 438-440.

Garcia-Bocanegra, I., Dubey, J.P., Simon-Grife, M., Cabezon, O., Casal, J., Allepuz, A., Napp, S., Almeria, S., 2010. Seroprevalence and risk factors associated with Toxoplasma gondii infection in pig farms from Catalonia, north-eastern Spain. Res Vet Sci 89, 85-87.

Gebremedhin, E.Z., Agonafi, A., Tessema, T.S., Tilahun, G., Medhin, G., Vitale, M., Marco, V.D., 2013. Some risk factors for reproductive failures and contribution of Toxoplasma gondii infection in sheep and goats of Central Ethiopia: A cross-sectional study. Res. Vet. Sci. 95, 894-900.

Geuthner, A.C., Koethe, M., Ludewig, M., Pott, S., Schares, G., Daugschies, A., Bangoura, B., 2014. Persistence of Toxoplasma gondii tissue stages in poultry over a conventional fattening cycle. Parasitology 141, 1359-1364.

Gilbert, R.E., Stanford, M.R., 2000. Is ocular toxoplasmosis caused by prenatal or postnatal infection?

The present document has been produced and adopted by the bodies identified above as author(s). In accordance with Article 36 of Regulation (EC) No 178/2002, this task has been carried out exclusively by the author(s) in the context of a grant agreement between the European Food Safety Authority and the author(s). The present document is published complying with the transparency principle to which the Authority is subject. It cannot be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

Br J Ophthalmol 84, 224-226.

Györke, A., Opsteegh, M., Mircean, V., Iovu, A., Cozma, V., 2011. Toxoplasma gondii in Romanian household cats: Evaluation of serological tests, epidemiology and risk factors. Prev. Vet. Med. 102, 321-328.

Havelaar, A.H., Haagsma, J.A., Mangen, M.J., Kemmeren, J.M., Verhoef, L.P., Vijgen, S.M., Wilson, M., Friesema, I.H., Kortbeek, L.M., van Duynhoven, Y.T., van Pelt, W., 2012. Disease burden of foodborne pathogens in the Netherlands, 2009. Int J Food Microbiol 156, 231-238.

IBM Corp 2011. IBM SPSS Statistics for Windows (Armonk, NY: IBM Corp.).

Iovu, A., Gyorke, A., Mircean, V., Gavrea, R., Cozma, V., 2012. Seroprevalence of Toxoplasma gondii and Neospora caninum in dairy goats from Romania. Veterinary Parasitology 186, 470-474.

Jacobson, R.H., 1998. Validation of serological assays for diagnosis of infectious diseases. Revue scientifique et technique 17, 469-526.

Jones, J.L., Dubey, J.P., Jones Jl Jones Jeffrey L. Parasitic Diseases Branch, D.o.P.D., Malaria, C.f.G.H.C.R.N.E.A.G.A.U.S.j.c.g., Dubey Jp Dubey Jp United States Department of Agriculture, A.R.I.A.R.S.B.M.D.U.S., 2012. Foodborne toxoplasmosis. Clinical Infectious Diseases 55, 845-851.

Jurankova, J., Basso, W., Neumayerova, H., Balaz, V., Janova, E., Sidler, X., Deplazes, P., Koudela, B., 2014. Brain is the predilection site of Toxoplasma gondii in experimentally inoculated pigs as revealed by magnetic capture and real-time PCR. Food Microbiol 38, 167-170.

Jurankova, J., Basso, W., Neumayerova, H., Frencova, A., Balaz, V., Deplazes, P., Koudela, B., 2015. Predilection sites for Toxoplasma gondii in sheep tissues revealed by magnetic capture and real-time PCR detection. Food Microbiol 52, 150-153.

Jurankova, J., Opsteegh, M., Neumayerova, H., Kovarcik, K., Frencova, A., Balaz, V., Volf, J., Koudela, B., 2013. Quantification of Toxoplasma gondii in tissue samples of experimentally infected goats by magnetic capture and real-time PCR. Vet Parasitol 193, 95-99.

Katzer, F., Canton, G., Burrells, A., Palarea-Albaladejo, J., Horton, B., Bartley, P.M., Pang, Y., Chianini, F., Innes, E.A., Benavides, J., 2014. Immunization of lambs with the S48 strain of Toxoplasma gondii reduces tissue cyst burden following oral challenge with a complete strain of the parasite. Vet Parasitol 205, 46-56.

Kijlstra, A., Jongert, E., 2008. Control of the risk of human toxoplasmosis transmitted by meat. Int J Parasitol 38, 1359-1370.

Kijlstra, A., Meerburg, B.G., Bos, A.P., 2009. Food safety in free-range and organic livestock systems: risk management and responsibility. J Food Prot 72, 2629-2637.

Koethe, M., Straubinger, R.K., Pott, S., Bangoura, B., Geuthner, A.C., Daugschies, A., Ludewig, M., 2015. Quantitative detection of Toxoplasma gondii in tissues of experimentally infected turkeys and in retail turkey products by magnetic-capture PCR. Food Microbiol 52, 11-17.

Lopes, A.P., Dubey, J.P., Neto, F., Rodrigues, A., Martins, T., Rodrigues, M., Cardoso, L., 2013. Seroprevalence of Toxoplasma gondii infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption. Veterinary Parasitology 193, 266-269.

Luft, B.J., Remington, J.S., 1992. Toxoplasmic encephalitis in AIDS. Clin Infect Dis 15, 211-222.

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

The present document has been produced and adopted by the bodies identified above as author(s). In accordance with Article 36 of Regulation (EC) No 178/2002, this task has been carried out exclusively by the author(s) in the context of a grant agreement between the European Food Safety Authority and the author(s). The present document is published complying with the transparency principle to which the Authority is subject. It cannot be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

Mancianti, F., Nardoni, S., D'Ascenzi, C., Pedonese, F., Mugnaini, L., Franco, F., Papini, R., 2013. Seroprevalence, detection of DNA in blood and milk, and genotyping of Toxoplasma gondii in a goat population in Italy. Biomed Res Int 2013, 905326.

Meerburg, B.G., Van Riel, J.W., Cornelissen, J.B., Kijlstra, A., Mul, M.F., 2006. Cats and goat whey associated with Toxoplasma gondii infection in pigs. Vector Borne Zoonotic Dis 6, 266-274.

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

Neto, J.O., Azevedo, S.S., Gennari, S.M., Funada, M.R., Pena, H.F., Araujo, A.R., Batista, C.S., Silva, M.L., Gomes, A.A., Piatti, R.M., Alves, C.J., 2008. Prevalence and risk factors for anti-Toxoplasma gondii antibodies in goats of the Serido Oriental microregion, Rio Grande do Norte state, Northeast region of Brazil. Veterinary Parasitology 156, 329-332.

Noordhuizen, J.P.T.M., Thrusfield, M.V., Frankena, K., Graat, E.A.M., 2001. Application of Quantitative Methods in Veterinary Epidemiology. Wageningen Pers, 2nd edition, Wageningen, NL.

Opsteegh, M., Teunis, P., Mensink, M., Zuchner, L., Titilincu, A., Langelaar, M., van der Giessen, J., 2010. Evaluation of ELISA test characteristics and estimation of Toxoplasma gondii seroprevalence in Dutch sheep using mixture models. Prev Vet Med 96, 232-240.

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

Pomares, C., Ajzenberg, D., Bornard, L., Bernardin, G., Hasseine, L., Darde, M.L., Marty, P., 2011. Toxoplasmosis and horse meat, france. Emerg Infect Dis 17, 1327-1328.

R Core Team 2014. R: A language and environment for statistical computing. (Vienna, Austria, R Foundation for Statistical Computing).

Reed, G.F., Lynn, F., Meade, B.D., 2002. Use of Coefficient of Variation in Assessing Variability of Quantitative Assays. Clin. Diag. Lab. Immun. 9, 1235-1239.

Sacks, J.J., Roberto, R.R., Brooks, N.F., 1982. Toxoplasmosis infection associated with raw goat's milk. J. Am. Med. Assoc. 248, 1728-1732.

Schimmer, B., Luttikholt, S., Hautvast, J.L., Graat, E.A., Vellema, P., Duynhoven, Y.T., 2011. Seroprevalence and risk factors of Q fever in goats on commercial dairy goat farms in the Netherlands, 2009-2010. BMC Vet. Res. 7, 81.

Spišák, F., Turčeková, Ľ., Reiterová, K., Špilovská, S., Dubinský, P., 2010. Prevalence estimation and genotypization of Toxoplasma gondii in goats. Biologia 65, 670-674.

Stormoen, M., Tharaldsen, J., Hopp, P., 2012. Seroprevalence of Toxoplasma gondii infection in Norwegian dairy goats. Acta Veterinaria Scandinavica 54, 75.

Talabani, H., Asseraf, M., Yera, H., Delair, E., Ancelle, T., Thulliez, P., Brezin, A.P., Dupouy-Camet, J., 2009. Contributions of immunoblotting, real-time PCR, and the Goldmann-Witmer coefficient to diagnosis of atypical toxoplasmic retinochoroiditis. J Clin Microbiol 47, 2131-2135.

Tassi, P., 2007. Toxoplasma gondii infection in horses. A review. Parassitologia 49, 7-15.

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. Toxoplasma gondii: from animals to humans Int. J. Parasitol. 30, 1217-1258.

Thiebaut, R., Leproust, S., Chene, G., Gilbert, R., 2007. Effectiveness of prenatal treatment for congenital toxoplasmosis: a meta-analysis of individual patients' data. Lancet 369, 115-122.

The present document has been produced and adopted by the bodies identified above as author(s). In accordance with Article 36 of Regulation (EC) No 178/2002, this task has been carried out exclusively by the author(s) in the context of a grant agreement between the European Food Safety Authority and the author(s). The present document is published complying with the transparency principle to which the Authority is subject. It cannot be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.

Tzanidakis, N., Maksimov, P., Conraths, F.J., Kiossis, E., Brozos, C., Sotiraki, S., Schares, G., 2012. Toxoplasma gondii in sheep and goats: seroprevalence and potential risk factors under dairy husbandry practices. Veterinary Parasitology 190, 340-348.

van der Giessen, J., Fonville, M., Bouwknegt, M., Langelaar, M., Vollema, A., 2007. Seroprevalence of Trichinella spiralis and Toxoplasma gondii in pigs from different housing systems in The Netherlands. Vet Parasitol 148 371-374.

van Engelen, E., Luttikholt, S., Peperkamp, K., Vellema, P., Van den Brom, R., 2014. Small ruminants abortions in the Netherlands during lambing season 2012-2013. Vet. Rec. doi: 10.1136/vr.102244.

van Knapen, F., Franchimont, J.H., van der Lugt, G., 1982. Prevalence of antibodies to toxoplasma in farm animals in the Netherlands and its implication for meat inspection. Vet Q 4, 101-105.

Vuong, Q.H., 1989. Likelihood ratio tests for model selection and non-nested hypotheses. Econometrica. 57, 307-333.

The present document has been produced and adopted by the bodies identified above as author(s). In accordance with Article 36 of Regulation (EC) No 178/2002, this task has been carried out exclusively by the author(s) in the context of a grant agreement between the European Food Safety Authority and the author(s). The present document is published complying with the transparency principle to which the Authority is subject. It cannot be considered as an output adopted by the Authority. The European Food Safety Authority reserves its rights, view and position as regards the issues addressed and the conclusions reached in the present document, without prejudice to the rights of the authors.