EFSA EXTERNAL SCIENTIFIC 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. Final report[1]

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ABSTRACT

Scientific literature was reviewed to obtain 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. Using a systematic review approach 1766 records were identified and screened. Data was extracted from 267 records that presented results with a direct detection method, and 75 records reporting risk factor analyses. Brain and heart were among the predilection sites in pigs, small ruminants, horses and poultry, but not in cattle. Based on the obtained information, tissues were selected for sampling in the

[1] Question No EFSA-Q-2015-00217.

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Suggested citation: Opsteegh M, Schares G and Joke van der Giessen on behalf of the consortium, 2016. Relationship between seroprevalence in the main livestock species and presence of *Toxoplasma gondii* in meat (GP/EFSA/BIOHAZ/2013/01) An extensive literature review. Final report. EFSA supporting publication 2016:EN-996, 294 pp.

Available online: www.efsa.europa.eu/publications

experimental phase of the project: heart and diaphragm was selected for pigs and horses; heart and drumstick/lower leg muscle for chickens; and liver and diaphragm for cattle. The information obtained from records that evaluate direct detection methods based on spiked samples was limited. By entering the data from studies providing a direct comparison of two or more direct detection methods into a performance matrix, it was clear that cat bioassay performs best, followed by mouse bioassay. PCR can perform similarly to mouse bioassay depending on sampling strategy and protocol details. Detection based on microscopy lacks sensitivity. Overall direct detection rates for seronegative and seropositive animals were calculated based on data from the publications that presented matched indirect and direct detection results. Indirect detection showed concordance with the detection of parasites by cat bioassay, mouse bioassay or PCR in pigs, small ruminants and a lack of concordance in cattle and horses. Most risk factor studies focussed on pigs and small ruminants. The presence of felids, the likelihood of fodder contamination and a low confinement level were generally associated with increased risk of *T. gondii* infection at the farm. Due to the limited number of available studies and antithetic outcomes in available studies the literature review provided no clear picture of the effects of the drinking water source and the likelihood of transmission via rodents on risk of *T. gondii* infection.

KEY WORDS

Toxoplasma gondii, seroprevalence, tissue cysts, risk factors, livestock, review

SUMMARY

Toxoplasma (T.) gondii, an intracellular coccidian parasite, is one of the most successful parasites worldwide. Felids are definitive hosts of this parasite where after infection sexual reproduction takes place in the intestinal tract 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 oocysts (e.g. when handling soil or cat litter, via water, or on unwashed vegetables) or tissue cysts in raw or undercooked meat. When a woman is infected for the first time during pregnancy, *T. gondii* can be transmitted to the fetus and result in abortion or a baby born with central nervous system abnormalities or chorioretinitis. *T. gondii* can also cause ocular toxoplasmosis after acquired infection in immunocompetent individuals and can lead to severe disease in immune-compromised 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. In order 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. Serological assays are commonly used to determine the prevalence of antibodies (i.e. exposure to *T. gondii* infection) but it is not clear what seropositivity means in terms of the presence of infective tissue cyst in various livestock species thus the risk of human infection. Theoretically there should be a strong correlation, as both antibodies and tissue cysts are assumed to persist life-long in sheep and pigs, but studies comparing indirect and direct detection methods are limited especially in cattle, where DNA of *T. gondii* has been detected in seronegative cattle. Information on the prevalence of infective tissue cysts by the main livestock 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. 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.

The overall goal of the project is to gain information and knowledge on the presence and infectivity of *T. gondii* cysts in meat and other edible tissues of the main meat-producing animals and its relationship with *T. gondii* seroprevalence in animals. In this report, we describe the results of the extensive literature search and review of available data on *T. gondii* in meat of the main livestock species (pigs, cattle, sheep, goats, chickens, turkeys and horses) using the systematic review approach but without performing meta-analyses. The main questions we studied are (1) the anatomical distribution of the cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals for optimisation of detection (2) the available methods for detecting the presence and infectivity of *T. gondii* cysts, including their sensitivity and specificity; (3) the relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues; and (4) risk factors for *T. gondii* infection in the main livestock species.

Two *a priori* protocols were designed: one for tasks that require studies based on direct detection of *T. gondii* (tasks 1, 2 and 3), and a separate one to study the risk factors (task 4). A total of 1766 records were identified and screened for relevance and eligibility. Data were extracted from 267 records for tasks 1, 2 and 3, and 75 records for task 4.

Papers that report results of direct detection methods for more than one tissue per animal, were used to study the anatomical distribution in the different livestock species. In order to rank the tissues according the presence of *T. gondii* , a total score was calculated that takes into account the ranking of a tissue within the study, as well as the fraction of studies in which the tissue tested positive. This was done for each animal species separately. Limited data were available for turkeys and horses. The top

5 ranked tissues varied by species but brain and heart were identified as predilection sites for pigs, sheep, chickens, turkeys and horses. Predilection sites in cattle are different from those identified in these other species and the scores for the top ranking tissues were low compared to the other species. Based on these results, tissues were selected for the experimental phase of the project. In pigs, horses and chickens the heart was selected as predilection site; in cattle the liver was selected. In cattle, pigs and horses the diaphragm was selected as representative of edible tissue; in chickens drumstick/lower leg muscle were selected.

The performance of available methods for detecting the presence of *T. gondii* was evaluated based on two types of studies. Firstly, data was extracted from papers that evaluated the performance of a direct detection method based on spiking experiments. These papers show that most PCR-based methods are able to detect a DNA concentration equivalent to one parasite. However, since this is mainly based on testing DNA dilution series, it provides limited information about the performance of these methods on samples from animals harbouring tissue cysts after natural infection. Secondly, data from papers that presented matched results with two or more direct detection methods for experimentally or naturally infectected animals were used to complete a performance matrix. This shows that cat bioassay performs best, followed by mouse bioassay. PCR can perform similarly to mouse bioassay depending on the sampling and protocol details. Detection based on microscopy lacks sensitivity.

To study the relationship between seroprevalence and presence and infectivity of *T. gondii* in meat and edible tissues, only studies reporting matched direct and indirect detection results for naturally infected animals were considered. As the relationship is influenced by the performance of the direct detection method, the dataset was further limited to include only results based on cat bioassay, mouse bioassay and PCR. The number of useful studies for turkeys, horses, cattle and goats was limited. Available data suggest poor to moderate overall concordance between detection of antibodies and presence of the parasite in pigs, small ruminants and chickens; and no or poor overall concordance in cattle and horses. However, concordance varied between individual studies and in pigs, sheep and chickens, direct detection rates in up to a 100% of seropositive animals (and 72% of seropositive goats) have been reported in literature, whereas a maximum direct detection rate of 10% was reported for seropositive cattle and horses.

The number of studies providing information on potential risk and protective factors in pigs and small ruminants was suitable; for cattle, horses and poultry there were almost no studies available. A total number of 75 references including a total number of 111 individual studies were analysed. Many studies reported that differences in *T. gondii* prevalence in farm animals were associated with the age or the gender of animals, the size of flocks/herds/farms or the geographic location of the flocks/herds/farms. Although variables related to age are important, as the risk of being exposed to *T. gondii* during lifetime increases with age, it is related to the individual farm animal and not to the entire farm. Similar to age, gender is related only to the individual animal.Flock/herd/farm size represents a general farm characteristic which most likely is related to *T. gondii* specific on-farm risk factors but gives no clear information on factors wich favour or prevent the transmission of *T. gondii*. Finally, the geographic location of a farm might be important because it is expected that, e.g. climatic effects related to the geographic location of a farm could influence the exposure to *T. gondii*; but again, unexplained geographic differences between farms provide no information on the underlying factors (e.g. climatic factors) responsible for these differences in exposure. Thus, data related to age or gender of animals, the size of flocks/herds/farms or the geographic location do not provide a base for the development of strategies to prevent *T. gondii* infection at farm level because most likely they have no direct effect on the risk of infection and should be regarded as confounders. Finally, only those variables for which it can be expected that they are biologically relevant were taken into account. Definitive host related variables (presence of cats or on farm detection of *T. gondii* oocysts) and factors that serve as indicators of possible fodder contamination were almost always associated with an increased risk of *T. gondii* positivity in farm animals (studies in pigs and small ruminants). A low level of confinement was in most studies associated with risk (studies in pigs, sheep), although especially in cattle and under certain circumstances in pigs a low level of confinement may confer protection. Variables suggesting a likely transmission via rodents were associated with risk (pigs, sheep). However, when variables suggested unlikely transmission via rodents, this revealed either risk or protection (pigs, sheep). Variables characterizing contamination of drinking water or management intensity revealed no clear effect.

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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, 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

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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.

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 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, 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

INTRODUCTION AND OBJECTIVES

Toxoplasma (T.) 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 aberrant 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, but with the possibility to develop chorioretinitis later in life. *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., 2011), 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 implement potential intervention measures.

To reduce the risk of humans to become infected with *T. gondii* either congenitally or post-natally it is essential to know potential risk factors associated with the infection of farm animals with the parasite. This knowledge is essential for the future implementation of Good Farming Practices (GFP) allowing the farmers to develop efficient and sustainable control measures against *T. gondii* infection for their farms.

The objective of this project is 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 the cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals for optimisation of detection,

2: the performance of available methods for detecting the presence and infectivity of *T. gondii* cysts, including their sensitivity and specificity,

3: the relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues, and

4: the relationship between the on-farm risk factors and *T. gondii* infection in pigs, cattle, small ruminants, poultry and horses.

This report is based on the results of an extensive literature review covering the subjects mentioned above including available data reported for the main livestock species (pigs, cattle, small ruminants, poultry and horses and other equids.

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 WPs 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 project. The 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. The first three tasks (anatomical distribution, performance of direct detection methods, and relationship between indirect and direct detection) of the extensive literature review were combined in one work package (WP2) and one *a priori* protocol (Appendix A), as all three relied on studies reporting results with a direct detection method. The *a priori* protocol for the fourth task (risk factors) was designed separately (Appendix B) and dealt with in a separate workpackage (WP3), as studies based on indirect detection methods alone are acceptable for this task.

In WP4 the results of the literature review were used to design the studies of the experimental phase of the project. The experimental studies in cattle and small ruminants (WP5), pigs (WP6), horses (WP7) and poultry (WP8) are reported in a separate report.

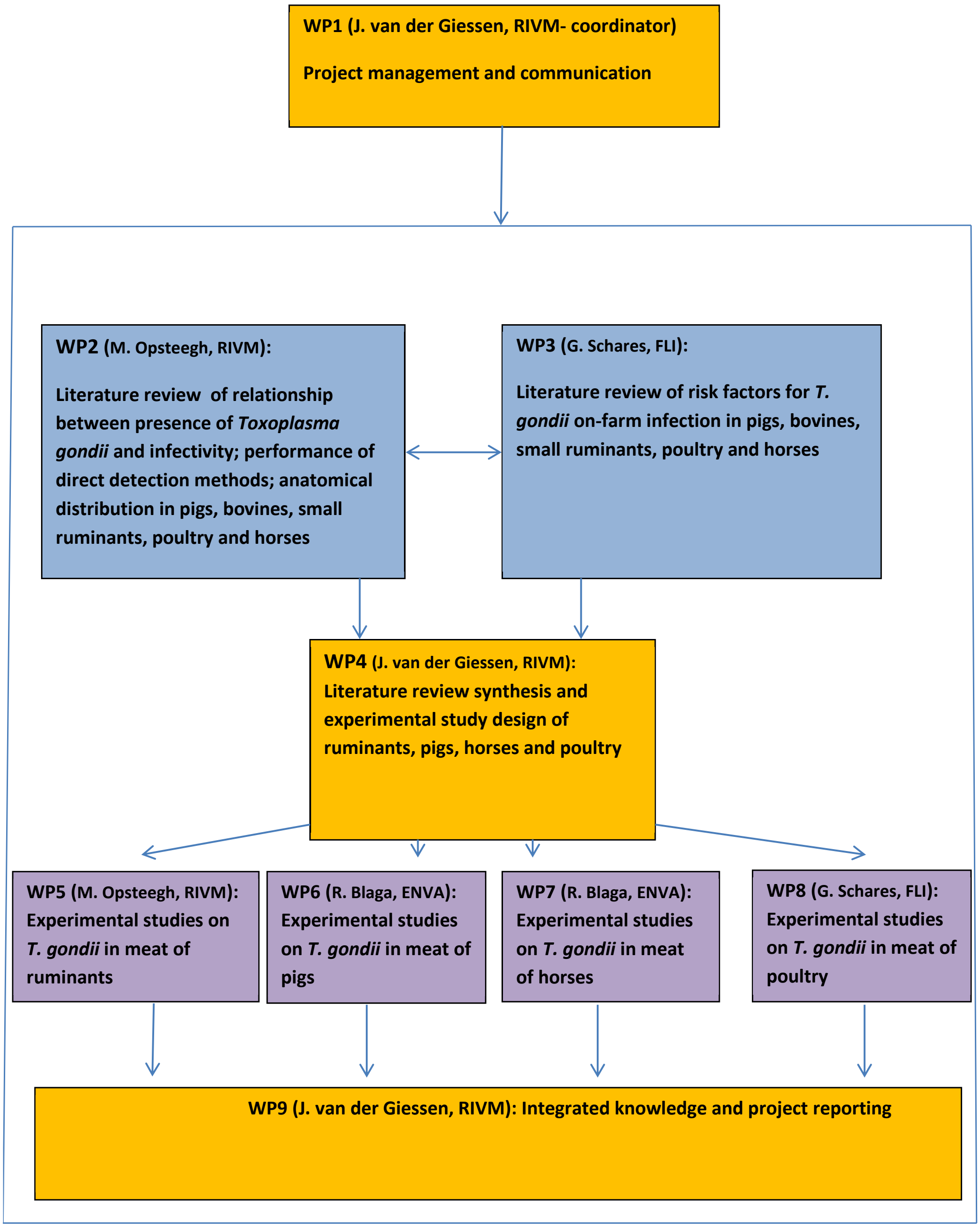

Figure 1: Project organisation and management

MATERIALS AND METHODS

1. Systematic review approach

The extensive literature review followed the systematic review approach using predefined *a priori* protocols based on Cochrane guidelines (http://handbook.cochrane.org/) and EFSA guidance (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/1637.pdf). The protocols included four main steps:identification, screening, data extraction and quality assessment.

2. Identification of relevant publications

2.1. Databases

Bibliographic searches were carried out using MEDLINE, EMBASE and BIOSIS.

Grey literature was not specifically searched for, but relevant documents were instead proposed by members of the consortium.

2.2. Search strategy

A search concept was designed to cover the following review questions:

- What is the anatomical distribution of the cysts in meat and other edible tissues? (Q1)
- What is the performance of available methods for detecting the presence and infectivity of *T. gondii* cysts in meat and other edible tissues? (Q2)
- What is the relationship between seroprevalence and presence and infectivity of *T. gondii* cysts in meat and other edible tissues? (Q3)

Specific search terms were developed to identify publications on the following key subjects:

- **Toxoplasma** as main topic/ pathogen of interest,

AND

- **animals** (pigs, cattle, small ruminants, poultry and horses) as target population

AND

- **detection** (method to detect infection or presence of cysts)

OR

- **presence (**antibody or *T. gondii* cysts)

A selection of known publication was checked against the retrieved records and it was noted that an important publication was missed (Dubey, 1983) (refid 1387), because it did not include any of the search terms for 'detection'or 'presence'. For that reason as an addition to the a priori protocol, search terms to cover all the tissues that may have been used to detect *T. gondii* were added (Appendix C):

OR

- **tissue**

To cover the review question 'What is the relationship between the on-farm risk factors and *T. gondii* infection in pigs, cattle, small ruminants, poultry and horses?' specific search terms were designed to identify publications on the following key subjects:

- **Toxoplasma** as main topic/ pathogen of interest,

AND

- **animals** (pigs, cattle, small ruminants, poultry and horses) as target population

AND

- **on farm risk factors**

The following technical items were also taken into account:

- UK and US spelling and terminology,
- Synonyms - e.g. cattle, cow, bovine, ruminants etc.
- thesaurus for subject searching (Medical Subject Headings system -'MeSH') articles indexed through controlled vocabulary
- Boolean operators (AND, OR, NOT),
- truncation (*) – e.g. Toxoplasm*
- and wild cards (#) – e.g. Toxopl#m*
- language restricted to English, German and French
- there was no limitation on publication date for the review questions on the relationship, performance and anatomical distribution, for the question on risk factors the search period will be limited to publications in last 20 years (i.e. publications from 1994 onwards) to address most recent knowledge in the topic.

Different combinations were tailored for each electronic database in order to narrow the amount of results retrieved but at the same time maximizing the number of relevant studies.

Retrieved records were imported in EndNote, and checked for duplicates. Next, records were imported into DistillerSR, a specific program for reference managing and evaluation. A second check for duplicates was performed using DistillerSR.

3. Screening of records

Initially, the selection protocol was validated for reliability and reproducibility, using a subset of publications already identified as either relevant or not relevant to the objective. Next, studies identified using the search strategy for bibliographic databases as well as those identified through thesis databases and identified grey literature were assessed against the inclusion and exclusion criteria for relevance and eligibility. Screening was performed in two stages. First, titles and abstracts were screened for relevance. Next, full-text reports of records found relevant were screened for eligibility.

3.1. Screening of titles and abstracts for relevance to the review question

All unique records were divided over the WP-members (2 reviewers per record), and after a quick title screen ("Is this record potentially relevant?") the relevance for screening of full text was determined based on title and abstract. If the first reviewer considered a record relevant, it was included in the full-text screening. When the reviewer did not consider the record relevant, the record was screened by the second reviewer. If the second reviewer considered the record relevant it was included in full-text screening, if not, the record was added to a list of non-relevant records. If no abstract was available or the abstract was too vague, the full text version was retrieved and screened. The titles and abstracts were screened for relevance using the following criteria:

Inclusion criteria WP2 and WP3:

- Peer reviewed scientific publications published or in press, or PhD/doctoral thesis
- Reports of original data as a primary source (e.g. remove reviews, editorials or letters to the editors without the original data)
- Paper addresses key elements in the review questions
    - Studies concerning the pathogen of interest (*T. gondii*, all isolates)
    - At least one of the animal species of interest is included. Host species: restricted to food animals most commonly consumed in Europe: pigs (domestic only), cattle (*Bos taurus* breeds), small ruminants (domestic sheep and goats), poultry (domestic chickens and turkeys) or horse and ponies.

Additional inclusion criteria WP2:

- At least one tissue (no restrictions on type of tissue) was tested using a direct detection method

Direct detection method: any direct detection method is accepted (e.g. cat or mouse bioassay, *in vitro* cultivation, PCR or antigen-ELISA or other method for antigen detection), publications that report results with only indirect detection methods are not (these may still be suitable for WP3).

Additional inclusion criteria (WP3):

- An assessment of risk or protective factors is presented

Exclusion criteria (WP3):

- Study published before 1994
- Case reports
- Risk/protective factors assessed based experimentally infected animals only
- Study contains no data driven assessment of on farm risk and protective factors
- Assessment is limited to risk or protective factors that are not applicable to European husbandry system (e.g. tropical climatic condition, non-European breeds)

3.2. Examining full-text reports for the eligibility of studies

Any of the inclusion and exclusion criteria (3.1) that could not be properly evaluated based on title and abstract alone were evaluated based on the full-text.

Based on conflicting answers in abstract screening it was noticed that the exclusion criterion for WP3 that focuses on non-European husbandry was open for different interpretations. The criterion was phrased: *Assessment is limited to risk or protective factors that are not applicable to European husbandry system (e.g. tropical climatic condition, non-European breeds).* This led some screeners to exclude studies from e.g. tropical countries, whereas other screeners only excluded those studies when e.g. climatic factors or non-exotic breeds were the only factors studied in those records (i.e. if e.g. age or the presence of cats was also assessed the study was still included). After discussion in the consortium, it was decided that it is preferred to completely exclude these studies because risk factors do not act on their own, but are influenced by the other factors present. Therefore this exclusion criterion was rephrased on the full-text screening form: *Study is NOT conducted under European husbandry conditions (NB not all studies from non-European countries should be excluded, only when the husbandry conditions are clearly different e.g. because of incomparable climatic conditions or exotic breeds).* Based on this, non-European epidemiological studies from other continents were only included (e.g. from North America, South America or Asia), if they had been performed on farms with husbandry conditions similar to husbandry conditions in Europe, with European breeds and under non-tropical climatc conditions, resembling those existing in different parts from Europe.

Additional exclusion criteria that are evaluated in this phase are:

- Full-text could not be obtained within two weeks after selection for full-text screening was completed for all records

- Publications contains only duplicated data

For WP2 eligibility of records for the three tasks was evaluated, and records that were initially identified as relevant for WP2, but were not applicable to any of the tasks were excluded. The criteria for the tasks were:

- *Anatomical distribution*: Are samples from naturally or experimentally infected animals tested using a direct detection method **and** are those samples defined? NB. Any type of definition is acceptable, e.g. by type of tissue/fluid, Latin names or common names for meat-cuts.

- *Test performance direct detection method*: Is the test performance (e.g. detection limit, sensitivity, specificity) of a **direct** detection method evaluated?

Relationship direct and indirect detection: Is an indirect (antibody) detection method used **and** are results using direct and indirect detection provided for the same species? NB. The two types of results do not necessarily have to be matched per individual animal, it is also acceptable when the prevalence is provided based on a direct and an indirect detection method. For the records that were initially identified as relevant for WP3 it was assessed whether population, exposure, comparator, outcome and study design (PECOS) are reported (Yes/No). As the reporting of the population being addressed (P) and the presence of a risk factor assessment (E) were already evaluated in previous forms, this WP3 specific form focuses on the identification of comparators, outcome measures and study design.

The comparators (C): Only studies were included, that considered at least one of the following reference scenarios against which the outcome or exposure could be compared

- controls – animals without disease or as a reference group in the study; or
- no exposure – animals with a lack of exposure to the factor of interest; or
- reference situation – animal status at a point prior to exposure to risk factors
- a cumulative effect (dose –relation) between level of risk factor and outcome

The main outcome or endpoint of interest (O): Only studies reporting on a strength of association or an impact (effect) of a particular risk or protective factor to infection with *T. gondii* were included (i.e. reporting only raw data for individual animals are excluded).

- Dichotomous outcome (e.g. Relative risk, RR; Odds ratio, OR; Risk difference, RD; Incidence rate, IR; Proportions for groups of exposed and non-exposed animals)
- Continuous outcome (e.g. Mean difference, MD; Number, mean and standard deviation or confidence interval for groups of exposed and non-exposed animals)

The study designs chosen (S): Only studies with a defined study design were included:

- case-control,
- cohort studies,
- cross-sectional and
- studies with hybrid design.
- Experimental field studies (e.g. vaccination as field trial within environmental risk factors)
- Other, define:

Two independent reviewers screened papers for completeness of reporting the PECOS characteristics. If both reviewers concluded that a study reported all data, the study was considered relevant for the data collection phase. If both reviewers found that the same characteristic is missing, the study was excluded from WP3 data extraction (the record might still be included for WP2). In case of disagreements or doubts, inclusion of the study was discussed with the WP-leader.

4. Data extraction

To limit the number of publications for WP2 three additional exclusion criteria were implemented before data extraction of the full publication.

- Direct detection of *T. gondii* is limited to pathology results for one or more tissues. Note: this concerns pathologic descriptions without direct detection of the parasite itself, e.g. tissue damage that is consistent with infection. In case pathology is combined with e.g. specific staining, the article should be included.

- Direct detection is limited to the following tissues/fluids: placenta, semen, abortion material, umbilical cord, reproduction organs, milk or undefined tissue pools. Exception: Papers using these tissues to test for the sensitivity/specificity of two direct detection methods (other than pathology) should be included.

- Publication is excluded after discussion with the WP-leader for a specified reason. For example: because the article does not contain quantitative results about the different study groups, but combined results are presented.

Data was extracted from all papers considered eligible. For each eligible study, data were collected and entered by one of the members of the work package. The data was verified by the WP leaders during the analyses of the data and discrepancies were resolved by the WP leader, if necessary, after discussion with the screeners. Since the standardised electronic forms in DistillerSR became very extensive, for WP2 a combination of DistillerSR and Microsoft Excel was used to collect the data. Data from DistillerSR was subsequently imported into Excel spreadsheets.

5. Quality assessment

5.1. Quality assessment of records used to evaluate the anatomical distribution of tissue cysts

To prepare an overview of the anatomical distribution, results from different studies were combined by tissue. For that reason, it was not feasible to present quality scores for the different studies with these data. Therefore, for this task, it was decided to exclude studies that are not qualified to provide information on the anatomical distribution of tissue cysts. Two categories of studies were excluded:

1. Studies that report direct detection results for only one type of tissue or pool of tissues were excluded from this task. This also excluded studies that report only one relevant tissue in addition to non-relevant fetal/aborted or neonatal tissues (as described in section 4).

2. Studies or groups of animals in which detection was more likely to be caused by the presence of tachyzoites than by tissue cysts are excluded, i.e.:

- experimental infections using the RH or S48 strain, as it is known that these strains are non-cystogenic, i.e. they do not form tissue cysts

- animals tested within 3 weeks after inoculation

As the ranking was done within the study by comparing tissues that have been tested with the same method, the quality of the detection method was unlikely to influence the ranking. When there are variations in protocol within one study, these variations are usually linked to the technical limitations of some tissues (e.g. not all tissues can be digested). Therefore, variation in the protocol within a paper was not used as a quality criterion. The number of tissues tested within a study is automatically taken into account in the ranking.

5.2. Quality assessment of records used to evaluate the test performance of direct detection methods

Two types of publications were included for this task and the data collected separately. Firstly, the data from records reporting results for samples spiked with a known amount or concentration of DNA or parasites were extracted. As these publications are quite different in nature, no quality criteria were applied to exclude publications, but all data were collected in a table with a row per publication and a column for comments. In this case, the type of samples used for the spiking experiments was considered irrelevant and not considered an exclusion criterium.

Secondly, data was collected from records in which two direct detection methods were compared on samples of the same animal. All records reporting results with two or more direct detection methods were selected from the database and evaluated for relevance. Records were excluded when:

- Comparison of direct detection methods was limited to methods of the same type (e.g. two different PCR targets).
- In the publication itself it was described that the results of a method are invalid, and after excluding this method, no comparison of two direct detection methods remains.
- The results were unclear or not matched on animal level.
- Performance of the methods was equal or only negative results were presented.

Other factors that may affect the quality of the data are indicated with or in the table (e.g. results based on naturally or experimentally infected animals).

The comparison with indirect detection methods can also give an indication of the performance of a direct detection method, but this information is collected to evaluate the relationship between direct and indirect detection and therefore not collected as part of the performance-task.

5.3. Quality assessment of records used to evaluate the relationship between direct and indirect detection methods

Data extraction was limited to studies that report matched indirect and direct detection results for naturally infected animals. For a record to be included, direct detection needs to be performed on a relevant tissue and using one of the three best performing direct detection methods identified in task 2. All records meeting these criteria were included in the tables. When possible quality issues remain, these are described in a separate column for comments.

5.4. Quality assessment of records used to evaluate the relationship between on farm risk factors and *T. gondii* infection

For WP3 the checklist contained the criteria described in Table 1. In parallel to data extraction reviewers were asked to answer questions regarding quality of the reported studies. To reflect differences in relative importance, individual criteria received a variable weight within the scoring system. The weight for each criterion had been determined by sending out the list of criteria to eight epidemiologists (RVC, FLI) asking to mark with a cross a variable number of criteria as regarded as the most important. A criterion marked by n=x epidemiologists as most important was given a weight of "1+x" within the scoring system. For each study reported on during data extraction the quality score (QS) was obtained by calculating the sum of all scores given per study. Answers on questions regarding quality were mandatory. A summary of the quality for each record is presented in the results tables. This will consist of a score as 'poor', 'average' or 'good' quality and any important issues. During data extraction from studies each criterion mentioned in Table 1 had to be judged (yes=1/no=0). In case of the criteria answered with 'yes' the weights were summed up to the final score of the study under examination. 33.3% percentiles were calculated for all the scores given to 111 studies reported in 75 references. Based on these calculated percentiles the scoring of studies was carried out into the categories 'poor', 'average' and 'good'.

Table 1: Checklist for quality appraisal (WP3) of studies and their weight in the scoring system

	Criterion	Weight
Methods		
Study design	Can the type of study be clearly identified?	2
	There is a clear rational for the selection of study units (farms, herds, flocks, groups, animals)?	3
	Is the sample size clearly described?	3
	Is the sampling strategy clearly described?	6
	Sampling-How was the selection of farms/herds/flocks/groups? - Random	2
	Sampling-How was the selection of animals within farms/herds/flocks/groups ? - Random	3
Variables	Production type - no information	-1
	Intensity - no information	-1
	Age group - no information	-1
Data sources	Are the sources of data and methods to assess or to measure potential risk or protective factors ("factors of interest", "explanatory variables", "predictors") clearly described?	8
Bias	All major sources of information bias are identified and acknowledged?	5
	All major sources of selection bias are identified and acknowledged?	6
	Is the time or the time period, when study was performed clearly stated?	3
	Is the location where the study was performed clearly described (If yes a number of options are offered)?	2
Statistical methods	Main confounders are addressed	7
	Main confounders considered - Age	4
	Main confounders considered - Farm size	1
	Main confounders considered - Farm type	1
	Other major potential confounders not considered	-1
	Is the independence of explanatory variables taken into account?	4
	Are potential interactions between explanatory variables addressed/analyzed	4
	Is the unit (individual animal, group, farm, ...) targeted and on which the dependent variable is based in the statistical analysis clearly described	7
Results		
	Study species described	1
	Summary estimates provided	4
	Group data provided	4
	Other type of data provided	1
Discussion		
	Is the objective of the study clearly described?	5
	Is this study clearly focused on on-farm risk and protective factors for *T. gondii* infection in farm animals	4

RESULTS

6. Identified, included and excluded records

Using the search strategy for relationship, performance and anatomical distribution 934 records were identified in Medline, and an additional 316 records were added after searching Embase and Biosis. 381 records were retrieved using the search strategy for the on farm risk factors in Medline and 71 in Embase and Biosis. However, only 48 out of these 381 Medline records were not already included based on the other search strategy. Therefore, the initial database consisted of 1369 records in total. Sixteen publications were submitted by consortium members and included as grey literature. No PhD databases were searched, as it was argued that useful data from these would have been published and would thus already be identified by the general screening.

During the analyses, it was noticed several relevant papers were not included in the search results. Therefore, an additional search was performed using the search terms to cover the subject “tissue”, in MEDLINE (Appendix C), resulting in an additional 144 references, and in EMBASE and BIOSIS, which together resulted in an additional 237 references. Therefore the final database contained 1766 records.

These 1766 records were screened and screening results with reasons for exclusion are presented in Fig. 2. A complete list of records with identification numbers (refid) and status is provided in Appendix F. Records have been moved to the quarantine at any point in the process, but the screening results already obtained for these records were removed from the DistillerSR exclusion report. Therefore, all quarantining appears to have occurred before title and abstract screening in the PRISMA flowchart (Fig. 2).

6.1. Records identified, included and excluded for WP2

A total of 363 records were checked for relevance to the different tasks of WP2, and 18 were excluded. To further limit the data extraction to the most relevant records, additional exclusion criteria were added as detailed in section 4. This resulted in exclusion of 77 records: 13 because only direct detection methods that are not specific for *T. gondii* were used (e.g. suggestive pathological changes observed by microscopy); 57 because direct detection was limited to non-relevant tissues and these were not used for the evaluation of a direct detection method; 16 were excluded for other reasons which made it impossible to extract useful data from the records. For nine records excluded at that stage, two exclusion criteria applied, which is why the numbers do not add up to 77. This resulted in 267 records of which data was extracted for WP2, 6 of these were also included for WP3. Specific inclusion criteria were later on applied in the preparation of the tables for the different tasks of WP2 (detailed in the chapters) and 95 of the included references remained unused for the tables in this report.

6.2. Records identified, included and excluded for WP3

82 publications were checked for WP3 eligibility, of which 75 were regarded as eligible for WP3 (6 of these were also included for WP2). Data was extracted of these 75 records.

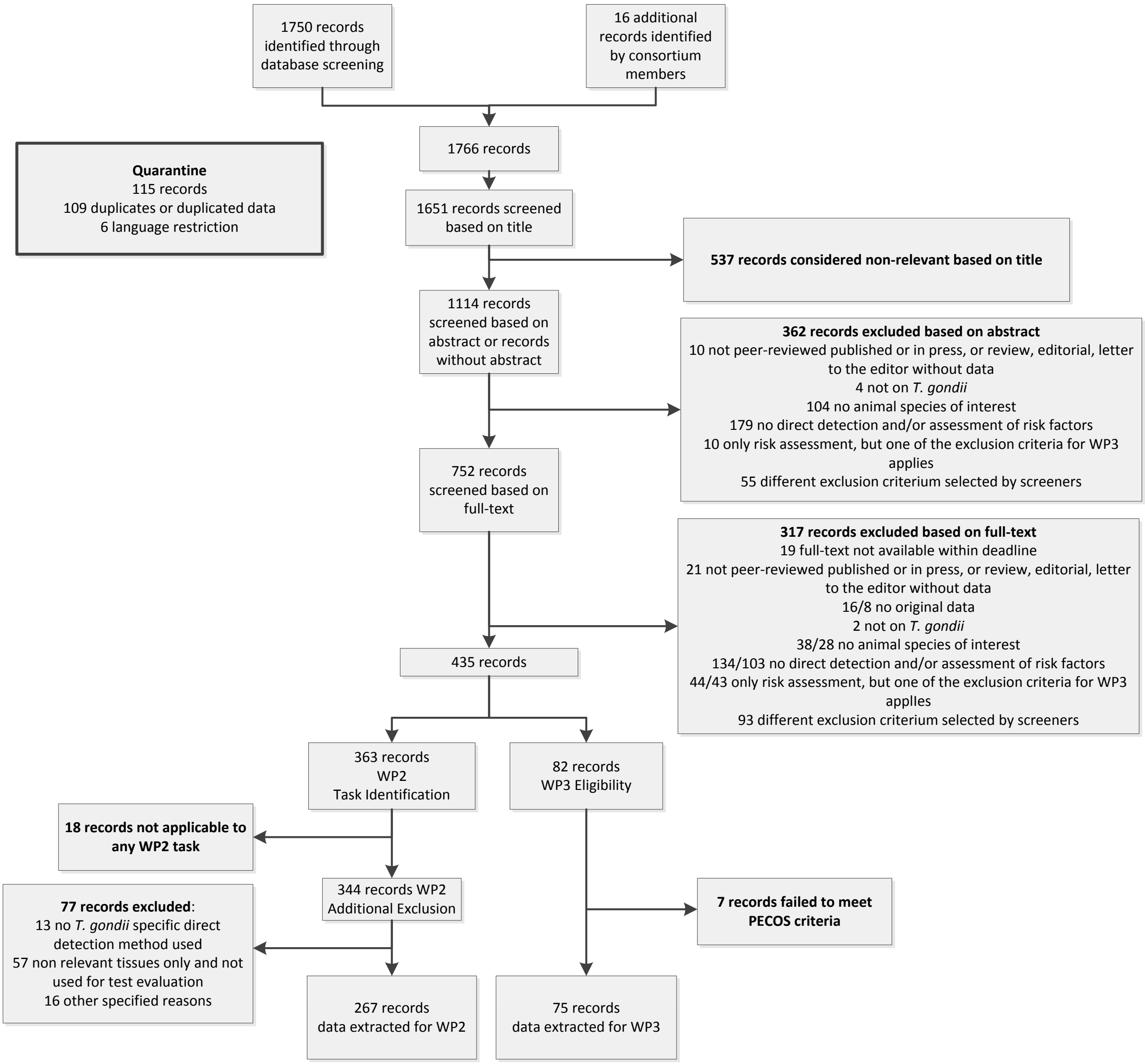

Figure 2: PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) with numbers of records identified, included and excluded, and the reasons for exclusions

7. The anatomical distribution of *T. gondii* tissue cysts in meat and other edible tissues

7.1. Introduction and aim

The overall aim of this task of the extensive literature review is to determine the anatomical distribution of *T. gondii* tissue cysts in meat of the main livestock species (pigs, cattle, sheep, goats, chicken, turkey and horses) to optimize the sampling choice(s) for slaughtered animals in the experimental phase. The first goal was to determine predilection sites per species, and from there, choose the optimal sample to study the correlation between serological results and the presence of tissue cysts. The second goal is to gain insight in the risk for consumers by focusing on edible tissues and to use this information to select a representative tissue to be tested in the experimental phase of the project.

7.2. General method for creation of the tables

Papers were grouped by livestock species. In case multiple species were discussed in one paper, the data of these species was extracted separately, thus allowing for analyses per livestock species. Subsequently, a first selection was made, taking out all studies that only investigated one type of tissue, or one tissue pool. Of the remaining papers the relevant data was extracted. When it was not possible to extract data per tissue, the paper was also excluded and the reason for exclusion was noted. When multiple direct detection methods were used in a paper or when there was a clear difference in experimental design (e.g. natural vs experimental infection), the data were extracted per separate study. In some papers all tissues tested negative with all direct detection methods that were used. When this concerned non-experimental infections (e.g. slaughterhouse animals), these papers were taken out, as it could be that all animals tested were true negatives. However, if multiple detection methods were used in a paper, of which at least one had positive results, all studies remained in the overview.

After data were extracted from all papers, the tissues were evaluated per study. From each study, tested tissues and the fraction of animals for which the tissue tested positive was recorded. Concerning edible tissues, various muscles and meat cuts have been tested for most animal species, but most of them only in a limited number of studies. Therefore, a combined score for muscles and meat (not including heart, diaphragm and tongue) was calculated to allow identification of the most-suitable representative of edible tissue. In case more than one meat or muscle tissue was tested in a study, the data from the original publication were used to obtain the number of animals that was positive for any one of the muscle or meat tissues. Since an animal is scored positive when any of the meat or muscle tissues is positive the combined meat and muscle score will be higher than the score for any of the individual muscle tissues in that study. In our opinion, the fraction of positives for the combined meat and muscles category provides the best indication of whether an infected animal poses a risk to consumers. However, as it is a combined category this is not a tissue that can be sampled, therefore a tissue with a comparable score (not necessarily an edible tissue) will be proposed for sampling as a representative of edible tissue.

After recording the fraction of positive animals per tissue, the tissues within each study were ranked according their level of positivity. First, the number of classes of positivity (including a class for negative) was determined (e.g. 5 classes) to ensure that tissues with equal positivity could be assigned the same rank (dense ranking). Next, the tissues were assigned a rank with the most positive tissue scoring 1-(1/#classes) (e.g. 4/5) and, for example, the third positive tissue scoring 1-(3/#classes) (e.g. 2/5). This way, the most positive tissue gets the highest score and this score will be closer to one when more classes of positivity are available within the study (e.g. 1-(1/7) > 1-(1/5)). Negative tissues always score 0 (e.g. 1-(5/5)). For each tissue, the average score was calculated: the 'within study score'. Note that the number of studies this average is based on varies per tissue. A hypothetical example of the calculation of the 'average within study score' is provided in Table 2.

Table 2: Calculation of "within study score", hypothetical example

Tissue	Study 1: Data	Study 1: Ranks	Study 2: Data	Study 2: Ranks	Study 3: Data	Study 3: Ranks	Average within study score
Heart	4/5	2/4	7/10	2/4	2/6	1/3	0.44
Brain	5/5	3/4	ND	ND	3/6	2/3	0.70
Liver	ND	ND	9/10	3/4	ND	ND	0.75
M. masseter	3/5	1/4	5/10	1/4	ND	ND	0.25
"Negative class"	NA	0/4	NA	0/4	NA	0/3	NA

In this example data are available from three studies. Together these studies provide information on heart, brain, liver and masseter but the individual tissues have not been included in all three studies (in "Data" column ND = not done). Per study, the fraction of animals for which the tissue tested positive by the total number of animals for which the tissue was tested is provided in the "Data" column; the negative class is not applicable (NA) for the "Data" column. In the "Ranks" columns, the tissues in a study are ranked by dense ranking with the most positive tissue scoring 1-(1/#classes). The ranks are averaged per tissue to calculate the "Average within study score".

Additionally, the percentage of studies in which a tissue scored positive was taken into account, but this percentage was weighted for the number of studies the tissue was tested in, i.e. the percentage was multiplied by the number of studies divided by the maximum number of studies for a tissue for that species: the weighted 'fraction positive studies'. For example: results for kidney were described in two studies with a positive result in one (p=0.50) and, for the species, results for heart were reported in the maximum number of studies (n=17, p=0.20). In that case, the score for kidney would be 0.50*(2/17)=0.06 and for heart 0.20*(17/17)=0.20.

The 'within study score' and 'fraction positive studies' were summed, and tissues were ranked based on this summed score. As the number of studies a tissue was tested in can have a substantial influence on the weighted 'fraction positive studies', the unweighted 'fraction positive studies' and the sum of within study score and unweighted 'fraction positive studies' are also presented.

7.3. Anatomical distribution in pigs

In total, 96 papers were included in WP2 for pigs. After exclusion of papers focusing on one tissue or tissue pool (48) or exclusion for other reasons, 32 papers remained. Of these, there were six papers that had results for two different direct detection methods, and one paper had results for three different detection methods. One paper concerned slaughterhouse animals that all tested negative (Wyss et al., 2000) (refid 429), and this paper was taken out, as these animals may have been true negatives. From three papers some animals were excluded as they were inoculated with non-cystogenic strains or they were killed within three weeks post infection. One paper was excluded as all animals died between 9 and 14 days post infection (Ito et al., 1974) (refid 1459). In total, 30 papers with 37 studies with direct detection methods multiple tissues have been compared (Appendix D, Table D1). Table 3 shows a summary of the results in porcine tissues.

Table 3: *T. gondii* detection in porcine 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]
brain	0.51	28	26	0.93	0.93	1.44	1.44
heart	0.60	20	20	1.00	0.71	1.60	1.32
meat/muscle combined	0.51	19	18	0.95	0.64	1.46	1.16
tongue	0.48	17	15	0.88	0.54	1.36	1.02
diaphragm	0.44	15	15	1.00	0.54	1.44	0.97
chorio-retinal coat	0.75	1	1	1.00	0.04	1.75	0.79
muscle pool *	0.67	2	2	1.00	0.07	1.67	0.74
hilar lymph node	0.67	1	1	1.00	0.04	1.67	0.70
ham*	0.53	4	4	1.00	0.14	1.53	0.67
thorax muscles/ribs*	0.46	5	5	1.00	0.18	1.46	0.64
thigh muscle*	0.60	1	1	1.00	0.04	1.60	0.64
left hind limb*	0.60	1	1	1.00	0.04	1.60	0.64
arm picnic*	0.48	2	2	1.00	0.07	1.48	0.55
limb muscle*	0.50	1	1	1.00	0.04	1.50	0.54
neck muscle*	0.50	1	1	1.00	0.04	1.50	0.54
belly muscle*	0.50	1	1	1.00	0.04	1.50	0.54
intestine mucosa+submucosa	0.50	1	1	1.00	0.04	1.50	0.54
loin/tenderlion (longismus)*	0.31	6	5	0.83	0.18	1.14	0.49
longissimus dorsi*	0.36	3	3	1.00	0.11	1.36	0.47
skeletal muscle*	0.37	3	2	0.67	0.07	1.04	0.44
right front limb*	0.40	1	1	1.00	0.04	1.40	0.44
liver	0.18	9	6	0.67	0.21	0.84	0.39
boston butt*	0.31	2	2	1.00	0.07	1.31	0.38
masseter muscle*	0.33	1	1	1.00	0.04	1.33	0.37
gastrocnemius muscle*	0.29	2	2	1.00	0.07	1.29	0.36
dorsal muscle*	0.30	2	1	0.50	0.04	0.80	0.34
lungs	0.26	5	2	0.40	0.07	0.66	0.33
bacon*	0.26	2	2	1.00	0.07	1.26	0.33
kidneys	0.15	6	4	0.67	0.14	0.82	0.29
shoulder loin*	0.25	2	1	0.50	0.04	0.75	0.29
bronchial lymph nodes	0.25	2	1	0.50	0.04	0.75	0.29
intestinal lymph nodes	0.25	2	1	0.50	0.04	0.75	0.29
eye(s)	0.25	2	1	0.50	0.04	0.75	0.29
small intestine	0.20	2	1	0.50	0.04	0.70	0.24
spinal cord	0.20	1	1	1.00	0.04	1.20	0.24
left front limb*	0.20	1	1	1.00	0.04	1.20	0.24
right hind limb*	0.20	1	1	1.00	0.04	1.20	0.24
tail	0.11	1	1	1.00	0.04	1.11	0.15
spleen	0.10	5	1	0.20	0.04	0.30	0.14
abdominal muscle*	0.00	2	0	0.00	0.00	0.00	0.00
scapular muscle*	0.00	1	0	0.00	0.00	0.00	0.00
intestine external muscle +serosa	0.00	1	0	0.00	0.00	0.00	0.00
mesenteric lymph nodes	0.00	1	0	0.00	0.00	0.00	0.00

	Average within study score	Number of studies		Fraction positive studies		Summed score	
		Tissue tested	Positive results	UW[a]	W[b]	UW[a]	W[b]
prescapular lymph node	0.00	1	0	0.00	0.00	0.00	0.00
pancreas	0.00	1	0	0.00	0.00	0.00	0.00
salivary gland	0.00	1	0	0.00	0.00	0.00	0.00

[a]UW: unweighted, [b]W: weighted

* These meat cuts and muscles were included in the “meat/muscle combined” category

In pigs, the anticipated predilection sites brain and heart rank at the top of the list, with tongue and diaphragm following closely. Many different muscles and meat cuts were tested and they generally rank high, even though they are often tested in a limited number of studies, which reduces their weighted scores. Notably, the combined meat and muscle category ranks third, indicating that, in infected pigs, *T. gondii* can usually also be found in edible tissue parts, thereby presenting a risk for consumers. Organs such as liver, lungs, kidneys, and spleen rank lower, even though they were tested in a reasonable number of studies. For research purposes, sampling of brain, heart, tongue or diaphragm are predilection sites that score similarly to the combined meat and muscle category. These tissues should therefore be considered as predilection sites but also when sampling to get an indication of infection in edible tissues.

7.4. Anatomical distribution in cattle

In total, 27 papers were included in WP2 for cattle. After exclusion of papers because they reported only on one tissue or tissue pool (12) or for other reasons, 13 papers remained. Three papers presented results for two different direct detection methods, and two papers presented results for three different detection methods. One paper described two different detection methods, for both calves and cows and results of these were separated into four different studies. Two papers were excluded, because there were only negative test results (refids 231 and 1257) (Dubey and Streitel, 1976; Fortier et al., 1990). One paper (refid 575) (Wiengcharoen et al., 2011) was excluded because all animals were inoculated with RH strain tachyzoites. From two papers some animals were excluded as they were inoculated with non-cystogenic strains or were killed within three weeks post infection. In total, for 10 papers with 19 studies multiple tissues have been compared (Appendix D, Table D2). Table 4: shows a summary of the results in cattle.

From Table 4 it is clear that small intestine and liver are predilection sites for *T. gondii* in cattle and skirt steak, lymph nodes, thigh muscle and top round steak have done very well in a limited number of studies. Out of liver and small intestine, liver is easier to collect and better suitable for bioassay in mice. Therefore, we propose to sample liver as a predilection site. As heart is often preferred as a sampling site and considered a predilection site, a direct comparison of all results for heart and liver is presented in Table 5. Although the data are limited, liver is positive in a larger fraction of studies and in most studies the fraction of positive animals is higher, thus supporting our decision to choose liver.
We propose to take diaphragm 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. Diaphragm performs less on the weighted summed score, as fewer studies were done, but in fact this score is high for the meat/muscle only because the data on different tissues from many studies were combined.

Table 4: *T. gondii* detection in cattle 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
muscle "top round"*	0.17	1	1	1.00	0.06	1.17	0.23
muscle "brisket"*	0.17	1	1	1.00	0.06	1.17	0.23
kidneys	0.05	10	2	0.20	0.13	0.25	0.18
muscle "roast" (semimembranosus and semitendinosus)*	0.08	3	1	0.33	0.06	0.42	0.15
mesenteric lymph nodes	0.08	6	1	0.17	0.06	0.25	0.15
eye(s)	0.08	5	1	0.20	0.06	0.28	0.14
thorax muscles/ribs*	0.05	5	1	0.20	0.06	0.25	0.11
lungs	0.05	8	1	0.13	0.06	0.18	0.11
spinal cord	0.04	6	1	0.17	0.06	0.21	0.10
loin/tenderloin (longissimus)*	0.04	4	1	0.25	0.06	0.29	0.10
spleen	0.03	7	1	0.14	0.06	0.17	0.09
psoas muscle*	0.00	3	0	0.00	0.00	0.00	0.00
gracilis muscle*	0.00	3	0	0.00	0.00	0.00	0.00
muscles from limbs and carcass*	0.00	1	0	0.00	0.00	0.00	0.00
prescapular lymph node	0.00	2	0	0.00	0.00	0.00	0.00
pancreas	0.00	2	0	0.00	0.00	0.00	0.00
adrenal glands	0.00	2	0	0.00	0.00	0.00	0.00
thyroid glands	0.00	2	0	0.00	0.00	0.00	0.00
salivary gland	0.00	2	0	0.00	0.00	0.00	0.00
thymus	0.00	2	0	0.00	0.00	0.00	0.00
uterus	0.00	1	0	0.00	0.00	0.00	0.00
colostrum	0.00	1	0	0.00	0.00	0.00	0.00

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

Table 5: Results (number of *T. gondii* positive animals by number of animals tested) for heart and liver per study

Study	Heart	Liver
639 (Lima Santos, 2010)_PCR	0/100[1]	ND*
919 (Esteban-Redondo, 1999)_Mousebioassay	0/10[2]	ND
919 (Esteban-Redondo, 1999)_Histology	0/5[3]	0/5[3]
919 (Esteban-Redondo, 1999)_PCR	0/10[3]	ND
1017 (Arias, 1994)_Mousebioassay	0/10	**5/10**
1046 (Dubey, 1993)_Catbioassay	**3/4**	**2/4**
1046 (Dubey, 1993)_Mousebioassay	0/4[3]	0/4[3]
1046 (Dubey, 1993)_Histology	0/4[3]	0/4[3]
1066 (Dubey, 1992)_Catbioassay	0/1[3]	0/1[3]
1251 (Costa, 1977)_Mousebioassay	0/5[4]	0/5[4]
1387 (Dubey, 1983)_Mousebioassay_calves	0/4	**3/4**
1387 (Dubey, 1983)_ Catbioassay_calves	**3/5**	**3/5**
1387 (Dubey, 1983)_Mousebioassay_cows	0/6[5]	0/6[5]
1387 (Dubey, 1983)_Catbioassay_cows	**1/3**	**2/5**
1398 (Beverley, 1977)_ Mousebioassay	0/9[6]	ND

*ND: not determined
[1]Brain: 2/100
[2]Brain: 1/10, psoas and gracilis muscle negative
[3]All tissues tested negative in these studies
[4]Liver, kidneys, heart and brain tested negative in this study, even though many other tissues tested positive (diaphragm, unspecified muscle, small intestine, spleen, lungs, unspecified lnn, eyes, testicle and blood)
[5]Many tissues tested negative, only for small intestine and mesenteric lnn 1 out of 6 tested positive.
[6]Only unspecified lnn positive (3/9); brain, heart and muscle negative

7.5. Anatomical distribution in sheep

In total, 74 papers were included in WP2 for sheep. After exclusion of papers because they reported only on one tissue or tissue pool (48), or for other reasons, 12 papers remained. Five of these remaining papers included results for two different direct detection methods. In total, for 17 studies multiple tissues had been compared (Appendix D, Table D3). From one reference (refid 1432) (Dubey and Sharma, 1980) four out of nine animals were excluded from the data analysis as these animals were killed and tested within three weeks after infection. None of the studies were performed using the RH or S48 strain. Table 6 shows a summary of the results in ovine tissues.

There is a striking similarity between the results for sheep and pigs. Again, brain and heart rank at the top of the list, closely followed by diaphragm. In contrast to pigs, tongue scores lower, but this may have been influenced by the fact that data are available from one sheep study only. Also for sheep, different muscles are found at the top of the list and the combined meat and muscle category ranks third. Spleen scores relatively high, but lungs, liver and kidneys rank lower and ranks are comparable to those found for pigs. In conclusion, *T. gondii* also readily disseminates to the edible tissues of sheep and presents a risk for consumers. Sampling of brain, heart, or diaphragm should be considered as predilection site and as a representative for edible tissue.

Table 6: *T. gondii* detection in ovine 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]
brain	0.43	15	13	0.87	0.87	1.30	1.30
heart	0.44	9	8	0.89	0.53	1.33	0.98
muscle/meat combined	0.44	11	8	0.73	0.53	1.17	0.97
skeletal muscle*	0.83	1	1	1.00	0.07	1.83	0.90
thorax muscles/ribs*	0.75	1	1	1.00	0.07	1.75	0.82
diaphragm	0.50	4	4	1.00	0.27	1.50	0.77
hind limbs muscles *	0.54	3	3	1.00	0.20	1.54	0.74
masseter muscle*	0.44	3	2	0.67	0.13	1.11	0.58
spinal cord	0.50	1	1	1.00	0.07	1.50	0.57
tongue	0.50	1	1	1.00	0.07	1.50	0.57
spleen	0.39	3	2	0.67	0.13	1.06	0.52
front limbs muscles*	0.40	1	1	1.00	0.07	1.40	0.47
lungs	0.31	3	2	0.67	0.13	0.98	0.44
small intestine	0.33	1	1	1.00	0.07	1.33	0.40
uterine lymph nodes	0.33	1	1	1.00	0.07	1.33	0.40
mammary glands	0.33	1	1	1.00	0.07	1.33	0.40
liver	0.23	4	2	0.50	0.13	0.73	0.36
dorsal muscle*	0.20	1	1	1.00	0.07	1.20	0.27
gracilis muscle*	0.11	4	2	0.50	0.13	0.61	0.25
pancreas	0.17	1	1	1.00	0.07	1.17	0.23
adrenal glands	0.17	1	1	1.00	0.07	1.17	0.23
uterus	0.17	1	1	1.00	0.07	1.17	0.23
psoas muscle*	0.10	4	1	0.25	0.07	0.35	0.17
mesenteric lymph nodes	0.08	2	1	0.50	0.07	0.58	0.15
kidneys	0.06	3	1	0.33	0.07	0.39	0.12
cervical lnn	0.00	1	0	0.00	0.00	0.00	0.00
eye(s)	0.00	2	0	0.00	0.00	0.00	0.00
salivary gland	0.00	1	0	0.00	0.00	0.00	0.00
blood	0.00	1	0	0.00	0.00	0.00	0.00

[a]UW: unweighted, [b]W: weighted

* These meat cuts and muscles were included in the “meat/muscle combined” category

7.6. Anatomical distribution in goats

In total, 31 papers were included in WP2 for goats. Of these, 21 were not included in the table because they were either only about one type of tissue/pool of tissues per direct detection method (20 papers), or because data could not be extracted per animal species (1 paper). From the remaining 10 papers data was extracted. This included one paper that had separate results for two studies, as it consisted of both natural and experimental data. One paper (record 827) was excluded as the only animal for which multiple tissues were tested had died within three weeks post infection (Sreekumar et al., 2004). Limiting to animals ≥21 dpi led to exclusion of some of the animals from two other references. There were no studies in which animals had been infected with non-cystogenic strains. In total, multiple tissues have been compared in 10 studies from 9 papers (Appendix D, Table D4). Table 7 shows a summary of the results in caprine tissues.

Table 7: *T. gondii* detection in caprine 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]
meat/muscle combined	0.65	7	7	1.00	1.00	1.65	1.65
kidneys	0.51	7	7	1.00	1.00	1.51	1.51
brain	0.60	6	6	1.00	0.86	1.60	1.45
heart	0.57	6	6	1.00	0.86	1.57	1.43
liver	0.41	7	7	1.00	1.00	1.41	1.41
skeletal muscle*	0.70	5	5	1.00	0.71	1.70	1.41
small intestine	0.89	1	1	1.00	0.14	1.89	1.03
salivary gland	0.89	1	1	1.00	0.14	1.89	1.03
mammary glands	0.89	1	1	1.00	0.14	1.89	1.03
diaphragm	0.57	3	3	1.00	0.43	1.57	1.00
spleen	0.49	3	3	1.00	0.43	1.49	0.92
muscles front limbs*	0.75	1	1	1.00	0.14	1.75	0.89
muscles hind limbs*	0.75	1	1	1.00	0.14	1.75	0.89
dorsal muscle*	0.75	1	1	1.00	0.14	1.75	0.89
pancreas	0.58	2	2	1.00	0.29	1.58	0.87
pool of brain and heart	0.67	1	1	1.00	0.14	1.67	0.81
lungs	0.49	2	2	1.00	0.29	1.49	0.77
cervical lymph nodes	0.56	1	1	1.00	0.14	1.56	0.70
tongue	0.50	1	1	1.00	0.14	1.50	0.64
mesenteric lymph nodes	0.44	1	1	1.00	0.14	1.44	0.59
spinal cord	0.39	2	1	0.50	0.14	0.89	0.53
pool of masseter and diaphragm*	0.33	1	1	1.00	0.14	1.33	0.48
thymus	0.33	1	1	1.00	0.14	1.33	0.48
unspecified lymph nodes	0.20	1	1	1.00	0.14	1.20	0.34
eye(s)	0.11	1	1	1.00	0.14	1.11	0.25
spinal fluid	0.00	1	0	0.00	0.00	0.00	0.00
adrenal glands	0.00	1	0	0.00	0.00	0.00	0.00
urinary bladder	0.00	1	0	0.00	0.00	0.00	0.00
testicle	0.00	1	0	0.00	0.00	0.00	0.00
blood	0.00	1	0	0.00	0.00	0.00	0.00

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

In goats, the anticipated predilection sites brain and heart rank high on the list. Interestingly, also kidneys and, as found in cattle, liver rank at the top of the list. Muscle tissues have high within study scores, and rank first when combined in the meat/muscle category. This shows the meat of infected goats presents a risk for consumers. For research purposes, sampling of kidneys, brain, heart or liver should be considered.

7.7. Anatomical distribution in chickens

In total, 60 papers were included in WP2 for chickens. After exclusion of papers because they were reporting only on one type of tissue/pool of tissues per direct detection method (38 papers), or because data could not be extracted per tissue or animal (2 papers), 20 papers remained. This included one paper that had separate results for three experiments. One paper was excluded, as it concerned only negative young chickens, and the adult chickens had only been tested for one tissue. From one paper (refid 595) some animals had to be excluded as they were killed within three weeks post inoculation (Yan et al., 2010). In total, for 21 studies from 19 papers multiple tissues have been compared (Appendix D, Table D5). Table 8 shows a summary of the results in tissues of chickens.

Table 8: *T. gondii* detection in tissues of chickens, 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	0.62	17	17	1.00	0.85	1.62	1.47
brain	0.46	20	18	0.90	0.90	1.36	1.36
Meat/muscle combined	0.37	14	13	0.93	0.65	1.30	1.02
pool of brain + heart + leg muscle	0.80	1	1	1.00	0.05	1.80	0.85
ovary duct	0.67	3	3	1.00	0.15	1.67	0.82
ovaries	0.61	4	4	1.00	0.20	1.61	0.81
ventriculus (gizzard)	0.75	1	1	1.00	0.05	1.75	0.80
pancreas	0.71	1	1	1.00	0.05	1.71	0.76
testicle	0.69	1	1	1.00	0.05	1.69	0.74
eye(s) – retina	0.58	3	3	1.00	0.15	1.58	0.73
spleen	0.51	4	4	1.00	0.20	1.51	0.71
limb muscle*	0.42	5	5	1.00	0.25	1.42	0.67
liver	0.38	6	5	0.83	0.25	1.22	0.63
pectoral muscle*	0.29	8	6	0.75	0.30	1.04	0.59
pool of brain + heart + pectoral muscle	0.38	3	3	1.00	0.15	1.38	0.53
lungs	0.34	4	3	0.75	0.15	1.09	0.49
proventriculus	0.43	1	1	1.00	0.05	1.43	0.48
skeletal muscle*	0.32	3	3	1.00	0.15	1.32	0.47
kidneys	0.28	3	3	1.00	0.15	1.28	0.43
intestine	0.26	3	2	0.67	0.10	0.93	0.36
pool of ovaries + oviducts	0.25	1	1	1.00	0.05	1.25	0.30
pool of heart + brain	0.17	1	1	1.00	0.05	1.17	0.22
bone marrow	0.08	1	1	1.00	0.05	1.08	0.13
eggs	0.06	2	1	0.50	0.05	0.56	0.11

[a]UW: unweighted, [b]W: weighted

* These meat cuts and muscles were included in the "meat/muscle combined" category

Also for chicken, the anticipated predilection sites heart and brain rank at the top of the list and the combined meat and muscle score shows that chicken meat presents a risk for consumer. In contrast to other species where reproductive organs generally rank low, ovary duct and ovaries rank high for chickens. Nonetheless, eggs are at the bottom of the list. The results for eggs are based on one paper (refid 1460) with a study in naturally infected chickens and one in experimentally infected chickens:

only one egg out of 327 eggs produced by 16 experimentally infected chickens was found positive (Jacobs and Melton, 1966). It is noteworthy that none of the tested tissues remained negative in all studies. For research purposes, sampling of heart or brain should be considered, as they score similarly to the combined meat and muscle category.

7.8. Anatomical distribution in turkeys

In total, five papers were included in WP2 for turkeys. In one study (Sedlak and Franti, 2000) wild turkeys (*Meleagris gallopova*) were used, but since there are very few reports on domestic turkeys available, results were included anyway. There were no data that had to be excluded because non-cystogenic strains were used or animals were killed within three weeks post infection. All five papers discussed multiple tissues tested with one direct detection method per paper and they were all included in the comparison (Appendix D, Table D6). Table 9 shows a summary of the results in turkeys.

Table 9: *T. gondii* detection in tissues of turkeys, 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	0.70	4	4	1	0.80	1.70	1.50
brain	0.46	5	4	1	0.80	1.26	1.26
limb muscle (drum stick)*	0.62	3	3	1	0.60	1.62	1.22
liver	0.40	5	4	1	0.80	1.20	1.20
meat/muscle combined	0.60	3	3	1	0.60	1.60	1.20
thigh muscle*	0.69	2	2	1	0.40	1.69	1.09
spleen	0.28	4	4	1	0.80	1.28	1.08
breast muscle*	0.46	3	3	1	0.60	1.46	1.06
proventriculus	0.45	3	3	1	0.60	1.45	1.05
kidneys	0.41	3	3	1	0.60	1.41	1.01
pool of heart + breast muscle + limb muscle	0.75	1	1	1	0.20	1.75	0.95
lungs	0.35	3	3	1	0.60	1.35	0.95
intestine	0.53	2	2	1	0.40	1.53	0.93
ventriculus (gizzard)	0.32	3	2	1	0.40	0.99	0.72
pancreas	0.31	2	2	1	0.40	1.31	0.71
colon	0.50	1	1	1	0.20	1.50	0.70
adrenal glands	0.50	1	1	1	0.20	1.50	0.70
oesophagus	0.50	1	1	1	0.20	1.50	0.70
testicle	0.21	2	2	1	0.40	1.21	0.61
blood	0.20	1	1	1	0.20	1.20	0.40
ovaries	0.00	1	0	0	0.00	0.00	0.00

[a]UW: unweighted, [b]W: weighted

* These meat cuts and muscles were included in the "meat/muscle combined" category

As the results for turkeys are based on such a limited number of studies the ranking is still unstable and can be affected by the availability of new results. Nonetheless, heart and brain also show up at the top of the list for turkeys and the combined meat and muscle score is high.

7.9. Anatomical distribution in horses

In total, seven papers were included in WP2 for horses. Two papers were excluded, as data could not be extracted per tissue or direct detection method. Only three papers discussed multiple tissues tested with one direct detection method per paper and they were all included in the overview (Appendix D, Table D7). No data from the horse studies had to be excluded for using non-cystogenic strains or because animals were killed within three weeks after infection. Table 10 shows a summary of the results in horses. As data are very limited, a second table that gives the results of the papers that use direct detection methods on single tissues is presented (Table 11).

All studies in Table 11 concern natural infections, and therefore, differences in the fraction of positives are more heavily influenced by differences in the prevalence of *T. gondii* infection than by the tissue used for detection. Therefore, this table does not help in selecting a tissue, other than confirming that positives have been found when brain or a pool of heart, diaphragm, spinal cord and oesophagus was used.

In conclusion, for horses information on the anatomical distribution is very limited. Based on the available studies heart appears to be a reliable choice for *T. gondii* detection.

Table 10: *T. gondii* detection in equine 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]
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
cerebrum	0.00	1	0	0.00	0.00	0.00	0.00
cerebellum	0.00	1	0	0.00	0.00	0.00	0.00
spleen	0.00	3	0	0.00	0.00	0.00	0.00
mesenteric lymph nodes	0.00	3	0	0.00	0.00	0.00	0.00
eye(s)	0.00	2	0	0.00	0.00	0.00	0.00
pancreas	0.00	1	0	0.00	0.00	0.00	0.00
stomach	0.00	1	0	0.00	0.00	0.00	0.00
adrenal glands	0.00	1	0	0.00	0.00	0.00	0.00

[a]UW: unweighted, [b]W: weighted

* These meat cuts and muscles were included in the “meat/muscle combined” category

Table 11: Summary of the studies that used a direct detection method on a single horse tissue

Tissue	Reference	Positive	Direct detection method	Positive status based on
Brain	(Evers et al., 2013) (refid 255)	14/398 (3.5%)	Mouse bioassay	Positive by mouse bioassay: tachyzoites, tissue cysts in brain
Serum	(Wang et al., 2011) (refid 583)	3/60 (5.0%)	Immunochromatographic strip (antigen ELISA)	Positive by antigen-ELISA and immunochromatographic strip
	(Wang et al., 2011) (refid 583)	3/60 (5.0%)	Antigen-ELISA	Positive by antigen-ELISA and immunochromatographic strip
Pool of heart, diaphragm, spinal cord, oesophagus	(Al-Khalidi and Dubey, 1979) (refid 1227)	2/24 (8.3%)	Mouse bioassay	Positive by mouse bioassay

7.10. Conclusions

To determine the anatomical distribution of tissue cysts, a summed score was calculated to take into account the ranking of the tissues within the applicable records and the fraction of studies in which the tissue tested positive. Species-specific conclusions are presented at the end of every section, to compare the different species, the number of records and top 5 ranking tissues for each species are summarised below (Table 12). These results point out that brain and heart, which are generally considered predilection sites, are among the top 5 tissues for all species except cattle. The other tissues vary by species. It also is clear that the summed scores, which can range from 0 to 2, are low for the top 5 tissues in cattle compared to the top 5 tissues of other species. The number of records available for turkeys and horses is very limited.

Table 12: Predilection sites for *T. gondii* in pigs, ruminants, poultry and horses

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

* The combined meat/muscle category or pooled tissues are not considered for inclusion in this table.

7.11. Selection of tissues for the experimental studies

One of the aims of the review of the anatomical distribution of *T. gondii* tissue cysts in meat and other tissues, was to select sampling sites for the experimental studies. Slaughterhouse studies will be performed in cattle, horses and chickens and for each species a predilection site and a representative of edible tissue will be sampled. The predilection site will be tested by mouse bioassay and the representative of edible tissue by MC-PCR. Based on the ranking of the combined meat and muscle category, a non-edible tissue often provides a better indication of the presence of *T. gondii* in edible tissues than testing of any one selected edible tissue. Therefore, we propose to use a representative tissue that that has a score very similar to the overall meat and muscle score. Table 13 shows an overview of the tissues that will be sampled in the experimental study designs for cattle, horses and chickens.

Table 13: Proposed sampling sites to represent a predilection site and edible tissue

Species	**Predilection tissue**	**Edible tissue representative**
Cattle	Liver	Diaphragm
Pigs	Heart	Diaphragm
Horses	Heart	Diaphragm *
Chicken	Heart	Drumstick and 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.

8. The performance of available methods for detecting the presence and infectivity of *T. gondii* tissue cysts

8.1. Introduction

T. gondii tissue cysts in meat are an important source of human infection. To develop prevention strategies, insight in the prevalence of *T. gondii* in different types of meat (e.g. different species, different cuts, or originating from animals in different husbandry systems) is needed. However, different techniques are available for detecting the presence of *T. gondii* tissue cysts, and depending on the characteristics of the method (e.g. discrimination of viable and non-viable parasites) and the performance (i.e. sensitivity and specificity) of the method the results obtained with different methods should be valued differently. The aim of this literature study is to provide an overview of the available direct detection methods and to evaluate their performance. The use of different methods in selected publications is identified, and a short description of identified methods is provided. Next, the available information on performance based on spiked samples and on comparison of two or more direct detection methods is summarized.

8.2. Overview of direct detection methods

In total 281 publications eligible for WP2 reported results with one or more direct detection method. As results for different species were entered separately this corresponded with 322 entries in the database. For each entry the screeners have reported the number of direct detection methods used in that entry: Most of the entries concerned results using only one method (n=181). However, 111 entries used two methods, 23 three methods, 5 used four methods, and 2 used five methods. This sums up to 502 entries with results based on a direct detection method.

An overview of used methods is presented in Table 14. Mouse bioassay and PCR are the most commonly used methods. Note that the search strategy and selection process focused on the main livestock species; it is possible that these methods are used in different proportions when human or other animal samples are tested.

Table 14: Frequency of direct detection methods in 502 entries of results (from 281 publications)

Method	Number of entries
Mouse bioassay	206
PCR	124
Microscopy without specific staining	59
Cat bioassay	52
IHC or IFAT	24
Antigen-ELISA (antibody-based detection of circulating antigens)	13 (4 publications)
Loop-mediated isothermal amplification (LAMP)	6 (4 publications)
In vitro isolation	3 (1 publication)
Other	15

8.3. Brief description of the direct detection methods

8.3.1. Cat bioassay

To perform a cat bioassay, cats without previous exposure need to be selected. This is usually done by demonstrating absence of antibodies using a serological assay (often modified agglutination test). The cats are fed up to 500g of meat or tissue to be tested for the presence of *T. gondii* tissue cysts. Subsequently, the cats are monitored for infection with *T. gondii* by testing their feces for oocysts (microscopy, PCR or bioassay in mice) for up to three weeks after exposure and tested for the presence of antibodies three weeks or longer post exposure. For further confirmation of infection *T. gondii* can be demonstrated in cat tissues (e.g. PCR or mouse bioassay).

Cat bioassay demonstrates oral infectivity of tissue cysts and enables testing of large portions of meat. Isolated oocysts can be used for strain isolation and genotyping. Cat bioassays can be ethically undesirable and costly.

8.3.2. Mouse bioassay

Depending on preference and type of sample, homogenates or artificially digested tissues are inoculated (usually intraperitoneally or subcutaneously) into mice. For digestion either acid-pepsin solution or trypsin is used, and differences in survival of bradyzoites and tachyzoites in these solutions have been suggested (Dubey, 1998). Usually 50 to 200g of tissue is digested and a fraction of the pellet is inoculated into mice, often between two and five mice per sample are used. Different mouse strains are used and additional immunosuppressive drugs may be administered to increase sensitivity. The mice are monitored clinically and, when mice die or need to be euthanized or at the end of the experiment, samples (e.g. brain, peritoneal fluid) are examined for the presence of *T. gondii* by e.g. microscopy or PCR. Usually, mice are additionally tested for the presence of antibodies against *T. gondii*.

Mouse bioassay demonstrates infectivity of *T. gondii*, but not necessarily confirms infectivity after oral ingestion. In particular, survival of trypsin digestion by tachyzoites, which are assumed to be less infective after oral ingestion, is a point of discussion. Mouse bioassays can also be used for strain isolation. Mouse bioassays are usually less costly than cat bioassays, but can also be undesirable for ethical reasons. Samples are smaller compared to cat bioassay, especially when taking into account that only a fraction of the digest is inoculated.

8.3.3. Detection of DNA using PCR

Several different targets are available for PCR-based detection of *T. gondii*; the B1-gene and the 529bp repeat element are the most common targets. All types of systems (conventional, nested, semi-nested and realtime PCR) have been described. In general, all of these methods can detect low concentrations of *T. gondii* DNA and the methods perform well on spiked samples or in case of disseminated toxoplasmosis. However, tissue cysts are sparse and commercial DNA isolation methods are usually designed for 25mg samples; the chance of detecting *T. gondii* in such a small sample is low. For that reason, the main limiting factor to the sensitivity of PCR-based detection of tissue cysts is the DNA isolation method. To enable testing of large samples and thereby increase sensitivity of the detection by PCR, methods based on artificial digestion, homogenisation and isolation over Percoll gradients and sequence-based magnetic capture have been described.

Detection of *T. gondii* DNA does not necessarily provide an indication of oral infectivity as non-viable parasites or tachyzoites, which appear to be less infective after oral ingestion, can also be detected. Development of viability PCRs is ongoing for various pathogens, however there are no such reports for *T. gondii* yet.

8.3.4. Detection of DNA using LAMP

In addition to PCR, LAMP-based DNA detection methods have been developed (refids 3, 328, 509 and 656) (Lin et al., 2012; Qu et al., 2013; Wang et al., 2013; Zhang et al., 2009) to overcome the need of an expensive thermocycler. On spiked samples, performance of these methods is often comparable to PCR. The method also has the same drawbacks as PCR concerning sample size and viability.

8.3.5. In vitro isolation

T. gondii tachyzoites can be cultivated in a wide range of cell lines. In vitro cultivation is commonly used to maintain or multiply parasites, e.g. for antigen preparation. After several passages in cell culture or mice strains may lose their ability to form tissue cysts or oocysts (non-cystogenic strains such as RH and S48).

Diagnostic usage of tissue culture based methods is limited and mainly described for fluid samples in which tachyzoites can be expected (e.g. liquor, peritoneal exudate, amniotic fluid). Meat homogenates or sediments from artificial digestion have been tested with variable success rates.

Only one study (refid 458) using a tissue culture based assay was included in WP2. In this publication culture based isolation is described for 10% of caprine, 7% of ovine and 4% of bovine milk samples (Dehkordi et al., 2013).

8.3.6. Microscopy

T. gondii tachyzoites (approximately 2 by 6 µm crescent-shaped organisms) or tissue cysts (an intracellular cluster of bradyzoites of up to 100 µm contained by a tissue cyst wall) can not be detected by macroscopic inspection of the meat but can be visualized under the microscope (Dubey et al., 1998a). Although parasites are visible with non-specific staining such as Giemsa or H&E, the use of specific staining with enzyme (IHC) or fluorescently (IFT) conjugated antibodies will help differentiation from other apicomplexan parasites or structures and increases sensitivity. Cross-reactivity of conjugated antibody, especially polyclonal anti-sera, can be an issue and specificity should be determined. Microscopy is labor-intensive and requires an experienced technician. The main disadvantage is size of the sample that can be examined. The use of microscopy directly on meat samples is limited, but it is often used secondarily to demonstrate infection in bioassay mice (tachyzoites in peritoneal fluid in acute infections or tissue cysts in brain in chronic infections).

8.3.7. Other direct detection methods

Four publications made use of detection of circulating antibodies (refids 269, 458, 507 and 583) (Dehkordi et al., 2013; Wang et al., 2011; Zhao et al., 2012a; Zhao et al., 2012b). In refid 269 and 507 chickens were tested using a commercial kit (Chicken toxoplasma circulating antigen (TCA) enzyme linked immunosorbent assay (ELISA) Kit (DRE73521, R&B Scientific, USA)). In refid 458 an in-house capture ELISA using rabbit anti-*T. gondii* IgG is developed and used to test milk samples of various species. Refid 583 describes the development of an immunochromatographic strip for on-site detection of circulating antigens in the blood of animals. They use a polyclonal antiserum derived from immunising sheep with tachyzoite-culture supernatant. Detection of circulating antigens is likely limited to the acute phase of infection when tachyzoites are replicating and disseminating throughout the body. The methods are likely to lack sensitivity in chronic infections with tissue cysts.

The 15 methods classified as other in Table 14 consisted of histopathology incorrectly classified as other in 6 entries, and a PCR-based method incorrectly classified as other in 2 entries. Detection in the remaining 7 entries was based on bioassay in 3 entries (one each in pigs (refid 1471), guinea pigs (Refid 1368) and 8 day old embryonated eggs (Refid 1368)), on parasitological examination without further details in 1 entry (refid 1312), on electron microscopy in 1 (refid 1436), on percoll-based isolation of tissue cysts and microscopy in 1 (refid 461) and IFT on a trypsin-digest in 1 (refid 1276).

8.4. Evaluation of direct detection methods based on spiked samples

In 16 publications and 27 entries the performance of a direct detection method was evaluated based on spiked samples (Table 15). In 25 entries this concerned a DNA-based method. In refid 938B a tissue culture based method is evaluated, and refid 1501 focuses on mouse bioassay.

In 20 entries, the spiking consisted of using a dilution series of *T. gondii* DNA in water, buffer or host DNA. The genome of *T. gondii* is 65Mbp and one parasite corresponds to 85fg of DNA (Khan et al., 2005). Depending on the PCR target, several copies may be present in the genome of one parasite; e.g. B1-gene has 35 copies (Burg et al., 1989), 529bp repeat element has 200-300 copies (Homan et al., 2000). Reported detection limits range from 1fg (509A) to 100pg (1024), but most of the PCR and LAMP-reactions included in Table 15 are able to detect *T. gondii* when DNA representing one parasite is present. DNA dilution series in water or buffer are useful for evaluating and comparing detection limits of PCR or LAMP reactions with different targets, however it has limited value when the detection of tissue cysts in meat needs to be determined. Firstly, as is clear from e.g. refid 1045 the PCR reaction will be less sensitive with an excess of host DNA present. Secondly, the efficiency of the DNA isolation will influence the performance of the complete detection method. Therefore, the evaluation based on spiking samples prior to DNA isolation is more useful in this context. Refids 625A, 767, 883, 887 and 938 demonstrate that in this case, the detection limit lies between 2.3 and 1000 parasites per gram or millilitre of sample. Meat samples do not contain individual parasites, but harbour tissue cysts and mature tissue cysts contain hundreds or thousands of bradyzoites. A concentration of one tissue cyst per 50g has been suggested. Therefore, in theory, when the detection limit based on spiked samples indicates that several parasites per gram are needed, one tissue cysts in a homogenized 50g sample might still be detectable. For a DNA based method, refid 938 is the only study that presents spiking experiments using tissue cysts. The results from refid 938 comparing spiking with single parasites (10^3) and spiking with tissue cysts (50 tissue cysts/g and 70 tissue cysts/g, corresponding to approximately $2.5x10^4$ and 3.5 $x10^4$ single parasites/g) suggest that there is additional loss of sensitivity when detection is aimed at tissue cysts rather than individual parasites. A possible explanation could be that the parasites present in tissue cysts are not as efficiently homogenised or the DNA extraction or invasion of tissue culture is less efficient for bradyzoites rather than tachyzoites.

The results from refid 938 for a tissue culture based detection method are not very different from their results for PCR ($5x10^3$ or $1x10^3$ parasites/g and 70 or 50 tissue cysts/g). However, this publication has the lowest sensitivity of PCR out of all studies that spiked samples with parasites before homogenisation and therefore, it is not clear from these results whether tissue culture does indeed have comparable sensitivity to PCR.

In refid 1501 the sensitivity of the mouse bioassay is determined using tissue cyst from mouse brain suspensions. The detection limit is around 0.0095 cysts injected into a mouse, but the number of bradyzoites per tissue cyst was not determined but assumed at 10^3-10^4, leading to the conclusion that 1 cyst per 100g sample should be detectable.

Table 15: Performance of direct detection methods for *T. gondii* based on the results with spiked samples

Refid	Assay	Spike[1]	Detection limit	Analytical specificity (cross reactions).
3A	qPCR, SAG1	genomic DNA equivalent to 10^{7}-10^{-2} tachyzoites per reaction	10fg (~0.1 tachyzoite)	blood samples from pigs prior to exp. infection with *T. gondii* were negative in qPCR
3B	LAMP, SAG1	genomic DNA equivalent to 10^{7}-10^{-2} tachyzoites per reaction	100fg (~1 tachyzoite)	blood samples from pigs prior to exp. infection with *T. gondii* were negative in LAMP
65	nested PCR, B1 gene	RH-strain DNA dilution series	first round: 17pg/ul second round: 170 fg/ul	No amplification with *Neospora caninum*, *Sarcocystis* spp., *Babesia ovis*, *Theileria annulata*, and healthy sheep genomic DNA
328A	RT-LAMP, 18S rRNA	CTG strain RNA dilution series (100ng to 10fg) 1g pork mixed with 100 tachyzoites, and 10-fold serial dilution of RNA isolate	10^{-7} (100fg) 3rd dilution (~1 tachyzoite in 1g pork)	No amplication was observed with RNA from *E. coli*, *Neospora caninum*, *Trypanosoma brucei*, *Cryptosporidium parvum*, *Toxocara canis*
328B	RT-PCR, 18S rRNA	CTG strain RNA dilution series (100ng to 10fg)	10^{-5} (10pg)	NA*
509A	qPCR, 529bp RE	RH strain DNA in water (1ng-0.1fg, tested in triplicate)	1fg	NA
509B	LAMP, 529bp RE	RH strain DNA in water (1ng-0.1fg, tested in triplicate)	10fg	NA
625A	MC-qPCR, 529bp RE	RH strain tachyzoites (31-250000) during homogenisation of 100g meat sample	95% detection limit (probit analysis): 227 tachyzoites per 100 g sample (95% CI: 107–3094)	NA
625B	qPCR, 529bp RE	RH strain DNA dilution series in water (500pg-2fg)	95% detection limit (probit analysis): 15.7 fg (95% CI: 10.0–55.9 fg) per PCR reaction	NA
629A	nested PCR, B1 gene	RH strain DNA dilution series in sheep DNA (3.2 pg/ml to 1.0 fg/ml estimated to correspond with 12-0.004 genome copies per µl)	50% detection limit (logistic regression) 0.02 parasite genome copies	sample:negative control ratio of 3:1, runs with contamination were rejected. There were no trends suggesting a build-up of contamination

Refid	Assay	Spike[1]	Detection limit	Analytical specificity (cross reactions).
629B	nested PCR, SAG1 gene	as 629A	50% DL 22 copies	as 629A
629C	nested PCR, 5' SAG2 gene	as 629A	50% DL 6 copies	as 629A
629D	nested PCR, 3' SAG2 gene	as 629A	50% DL 9 copies	as 629A
629E	nested PCR, SAG3 gene	as 629A	50% DL 6 copies	as 629A
656A	LAMP, 529bp RE	RH strain DNA dilution series 1ng-10fg	1pg/reaction	*Neospora caninum*, *Babesia gibsoni*, *B. bovis*, *Cryptosporidium parvum*, *Trypanosoma brucei* and *Theileria parva*.
656B	PCR, 529bp RE	RH strain DNA dilution series 1ng-10fg	10pg/reaction	NA
767	PCR, 529bp RE	RH strain tachyzoites (10^7-10^{-1}/ml) added to brain, muscle samples or TE buffer samples	100/ml of brain 10/ml muscle 1/ml TE	samples from two uninfected pigs were negative
883	PCR, B1 gene	ground mouse brain suspension with 10^5 to 10^0 tachyzoites/ml	10^2 parasites/ml of tissue suspension	NA
887	qPCR, ITS1	duplicate DNA dilution series 100ng-10fg from 3.7 x 10^5 to 3.7 bradyzoites added to 1g of pig muscle	100fg ~4 bradyzoites/g (but Ct of 46.54 and not a nice curve)	*N. caninum*, *H. hammondi*, *Eimeria acervulina*, *Eimeria tenella*, *Cryptosporidium parvum*, *Sarcocystis muris*, *Sarcocystis tenella*, *Sarcocystis cruzi* and negative control pigs
938A	nested PCR, P30	RH strain trophozoites (3.6 x 10^5/g to 0.5/g and 0/g) in homogenised meat sample RH strain tissue cysts 140/g to 10/g and 0/g in homogenised meat sample	10^3/g 50/g	NA
938B	tissue culture, 100mg meat suspension on HEL cells	RH strain trophozoites (3.6 x 10^5/g to 0.5/g and 0/g) in homogenised meat sample RH strain tissue cysts with between 10^2 and 10^3 parasites per cyst) 140/g to 10/g and 0/g in homogenised meat sample	5 x 10^3/g 70/g	NA
950	nested PCR, B1	10^6-0 tachyzoites added to 500mg powdered placenta tissue	10 parasites/500mg	*Sarcocystis tenella*, *S gigantea* and *Neospora caninum* DNA and *Chlamydia psittaci*-infected placental samples.

Refid	Assay	Spike[1]	Detection limit	Analytical specificity (cross reactions).
1024	PCR, SSU rRNA	RH strain DNA (1000ng-100pg)	100pg genomic DNA	One set of primers (TGc and TGd) also cross reacted with other DNA templates from *Sarcocystis* and sheep.
1045	PCR, 18S rRNA (4 primers to be used in combination with one of six universal primers)	RH strain DNA in water (100pg-10fg) RH strain DNA in host DNA (10pg)	100fg 10pg was positive, no other dilutions tested	human, canine, feline, porcine, bovine DNA, *S. cruzi*, *E. ahsata*, *E. vermiformis*, *E. coli* DNA PCR control samples lacking template DNA were included with each set of PCR's
1501	mouse bioassay	950-$9.5x10^{-4}$ cysts (from mouse brain suspension) inoculated, 2 mice per dilution	$9.5x10^{-2}$ positive in both mice, $9.5x10^{-3}$ positive in one mouse, $9.5x10^{-4}$ negative. Under the assumption of 10^3-10^4 bradyzoites per tissue cyst they conclude that 10 bradyzoites/g mouse brain or about 1 cyst/100g of tissue can be detected	NA
1540A	qPCR, SAG1	RH strain DNA dilution series equivalent to 10^7-10^{-1} parasite	1 tachyzoite	*Neospora caninum*, *Escherichia coli*, *Babesia bovis*, *Trypanosoma brucei*, *Cryptosporidium parvum*, and *Toxocara canis*.
1540B	PCR, SAG1	DNA dilution series equivalent to 10^7-10^{-1} parasite	100 tachyzoites	NA

[1] If the matrix to which the spike was added is missing from these cells, the information was not provided in the reference.
*NA: not available

8.5. Evaluation based on comparison of direct detection methods

All entries that reported results with two or more direct detection methods were filtered from the complete database, resulting in 126 entries. The manuscripts for these entries were checked to see if the used direct detection methods can be compared based on the presented results for naturally or experimentally infected animals. Fifty-three entries were excluded because the direct detection methods belonged to the same category (5 entries: 1 comparing circulating antigen techniques (refid 583), 2 comparing PCR protocols (refids 629, 1540), 1 comparing IFT on different sample preparations (refid 1276), and 1 using microscopy with different non-specific staining techniques (refid 1586)); because the results were not-matched or unclear (52); because only non-relevant tissues were used for the comparison (5); because the methods performed equally (8 entries; refids 271, 625, 822, 1110, 1213, 1424, 1459 and 1531) or all samples were negative in both methods (2), and 1 record was excluded because one of the results with one methods were considered invalid by the authors (refid 746) (Reitt et al., 2007). The remaining 73 entries were included in the relevant cells of a performance matrix (Table 16). The direct detection methods from these entries were categorised as: mouse bioassay, cat bioassay, PCR, LAMP, unspecific microscopy, microscopy using IHC or IFT, *in vitro* isolation, and detection of circulating antigens using e.g. ELISA. Other methods are excluded from this analysis. Although there will be variation between methods within a category (as detailed in section 8.3), which can influence the performance, lumping is needed to have a reasonable number of entries per cell. The method that scored the highest number of positives in a two-by-two comparison of methods from different categories was considered best. In many cases, the results from different groups of animals were combined to form an overall conclusion of the best method in the publication, but naturally and experimentally infected animals were kept separated. Results based on naturally infected animals are presented in green and those for experimentally infected animals in black. This differentiation has been made, as the true status of *T. gondii* infection is not known for naturally infected animals, therefore this evidence could be valued weaker than results based on experimentally infected animals. When results for more than two direct detection methods are compared, the entry was entered more than once in the matrix.

For each direct detection method the number of times the method scored better or worse is counted and the difference was calculated (Table 17). Methods were sorted by rank in the performance matrix.

Table 16: Performance matrix based on 73 comparisons of direct detection methods for *T. gondii* described in literature. Total number of comparisons with reference IDs per cell. Reference IDs printed in green when based on naturally infected animals and in black when based on experimentally infected animals.

Better / worse	CBio	MBio	LAMP	*in vitro*	PCR	Ag	IHC/IFT	microscopy	SUM
CBio		2: [947][999]	0	0	0	0	0	0	2
MBio	8:[708][835][1003] [1046][1146][1325] [1422][1387]		0	0	5:[494][589][694] [769][1409]	1: [269]	0	1: [1467]	15
LAMP	0	0		0	2: [3][509]	0	0	0	2
in vitro	0	0	0		0	0	0	0	0
PCR	3: [458] [757] [757]	10: [42][255][523] [555] [626][767] [883][919][1415] [950]	3: [328] [509] [656]	1: [458]		0	0	1: [919]	18
Ag	1: [458]	0	0	1: [458]	1: [458]		0	0	3
IHC/IFT	0	4:[231][962][1044] [1465]	0	0	3: [429][575] [716]	0		0	7
microscopy	2: [1146][1325]	12: [42][626][649] [767][883][1315] [1071][1146][1345] [1433][1465][1237]	1: [656]	0	8: [42] [656] [767] [770][883] [1484] [941][1646]	0	3:[467] [1396][1475]		26
SUM	14	28	4	2	19	1	3	2	73

Table 17: Summary of the results from the performance matrix

	Number of comparisons	Better	Worse	Difference
CBio	16	14	2	12
MBio	43	28	15	13
LAMP	6	4	2	2
In vitro isolation	2	2	0	2
PCR	37	19	18	1
Ag-ELISA	4	1	3	-2
Microscopy with IHC/IFT staining	10	3	7	-4
Non-specific microscopy	28	2	26	-24

Even though different cat or mouse bioassay protocols have been lumped into one category, tables 16 and 17 show that cat bioassay and mouse bioassay are the best performing direct detection methods, with cat bioassay usually outperforming mouse bioassay when these two methods are directly compared. Limited data are available for LAMP (6 comparisons), *in vitro* isolation (2 comparisons) and detection of circulating antigen (4 comparisons) and for that reason the ranking of these methods is unreliable. Microscopy with specific staining (IHC or IFT) and non-specific microscopy, regardless of protocol variation, are the least sensitive methods, with IHC/IFT outperforming non-specific microscopy in the three records that directly compare these two methods. PCR-based methods are commonly found on either end of the diagonal in the performance matrix. Looking in more detail, PCR-based methods never performed better than cat bioassay (3 comparisons), always performed better than IHC/IFT (3 comparisons), and usually performed better than non-specific microscopy (8 vs. 1 comparisons). However, the performance in comparison to mouse bioassay is not clear. There are lots of different PCR-based methods and there is variation in sample size, DNA isolation method, PCR-target as well as type of PCR (e.g. single round or nested conventional PCR and realtime PCR), and in this case protocol variation may be influencing the ranking, therefore a more detailed comparison of PCR-based methods versus mouse bioassay is provided in 8.6. Considering cat bioassay, mouse bioassay, IHC/IFT and non-specific microscopy there are a few entries with results that are not in agreement with the general picture (cells shaded in grey in the performance matrix). The details of these studies are discussed below.

In record 947 and 999 mouse bioassay performs better than cat bioassay. In **record 947** (Dubey et al., 1998b) experimentally infected pigs were tested. In this study mouse bioassay consisted of separate acid pepsin digestion of 50g of tongue, diaphragm and brain. Each digest was inoculated into ten mice. Two months after inoculation, mice were checked for a serological response by MAT and brains were examined microscopically and, if negative, bioassayed in mice. For cat bioassay, approximately 500g of skeletal muscle from the hind quarters per pig was fed to one cat and feces of the cats were checked for oocyst shedding by microscopy and bioassay in mice. Out of 17 pigs tested using both methods, three were negative in both and 10 were positive in both, but three tested positive by only mouse bioassay whereas one tested positive by only cat bioassay. More tissue has been tested in the mouse bioassay in this study (3 tissues and 10 mice per tissue) than is generally used which explains the higher sensitivity than cat bioassay in this study. When the results for only one of the tissues tested in mouse bioassay are considered, cat bioassay performs better.
The same is true for **record 999** (Dubey et al., 1996). In this case, acid pepsin digest of 50g of tongue, heart and brain of experimentally infected pigs is inoculated in 10 mice each; and remaining tongue, heart and brain made up to 500g using hind quarter muscles per pig was fed to one cat. Out of 41 pigs tested, there was one pig with discordant results and in this case mouse bioassay was positive and cat bioassay was negative.

In **record 1467** (Koestner and Cole, 1961) histopathology performs better than mouse bioassay. Results for experimentally infected sheep and experimentally and naturally infected cattle are described. Congenital cases are also described, but these results were not considered in our evaluation. As *Neospora caninum* was not known at the time, it can be questioned whether the three naturally infected cattle are indeed infected with *T. gondii* as all identification is based on morphological appearance. Results are presented separately for animals that died within 10 d.p.i and after 10 d.p.i.. For the five sheep that died within 10 days, three were positive by mouse bioassay and it seems that *T. gondii* was observed in histopathology for all five sheep. For the nine sheep that died more than 10 days post infection, *T. gondii* was recognized by histopathology in eight whereas mouse bioassay was positive for four. All three experimentally infected calves that died within 10 days were positive in histopathology, two of which were also positive in mouse bioassay. Three naturally infected cattle and seven experimentally infected calves that died more than 10 days after infection were negative in mousebioassay and histopathology. Based on these results it was concluded that histopathology performed better than mouse bioassay in this study. The methods are not clearly described; nonetheless there are some specifications that may explain the lack of sensitivity of mouse bioassay in this study. Mouse bioassay is performed by grinding tissue in saline and inoculating directly into mice. This means that no digestion was performed and therefore the size of the sample inoculated into the mice will be limited. The number of mice is not specified. Detection of infection in mice is based on non-specific microscopy on the mice, which is less sensitive than serology or PCR as is currently common. Fifteen to twenty-five sections of CNS were prepared for microscopy. Pictures of observed *T. gondii* parasites are presented in the paper, but it is not possible to completely verify identification based on histopathology alone.

In **record 919** non-specific microscopy performs better than PCR. This study describes non-specific microscopy, PCR and mouse bioassay results for sheep and calves experimentally infected with 10^3 or 10^5 oocysts and euthanized 6 weeks or 6 months post infection. In addition, parasitaemia is monitored using PCR, but these results are not considered in our comparison. All calf tissues tested were negative in histopathology and by bioassay. One calf tested positive by mousebioassay (10^5 oocysts, 6 weeks post infection, brain sample). Most sheep were positive in all assays. However, one sheep (10^3 oocysts, 6 weeks post infection) was negative in mousebioassay and PCR although cyst-like structures were identified in skeletal muscle. Therefore, mousebioassay and non-specific microscopy were considered to perform equal in this study, and both of these assays were considered to perform better than PCR. However, it needs to be stressed that the differences between the assays are small in this study, and confusingly, in the abstract the authors conclude that ‘PCR has more sensitivity and specificity when detecting the presence of *T. gondii* in large animals than histological detection’ which suggests (this is not explicitly stated) that the authors consider the additional positive sheep in histology a non-specific finding.

8.6. Comparison of mouse bioassay and PCR

From the performance matrix (Table 16) it can be seen that 15 publications present results for PCR and mouse bioassay. In 10 publications the mouse bioassay performs better, in 5 others PCR performs better. In addition, there was one reference (refid 625) excluded from the performance matrix as PCR and mouse bioassay performed equally in this study. As PCR would be a useful alternative to mouse bioassay if a similar sensitivity can be reached these studies are examined in more detail to find out whether certain characteristics of the PCR-based methods are correlated with a good performance. In two (42 and 1415) out of the 16 publications included in Table 18 the methods (especially the PCR) are not described clearly enough to identify influential details, and in 1 publication (refid 626) it is likely that mostly true negatives have been tested (1/48 samples MBio positive, no PCR positives) which is not suitable for method comparison. In refid 625 the sample size was similar for mouse bioassay and PCR (using sequence-based magnetic capture) and the performance of the methods is equal. In refids 919, 950 and 1409 results are also nearly equal for mouse bioassay and PCR and the same small samples have been tested in these publications. For the remaining 9 publications the sample used for PCR was smaller and in 5 of these publications mouse bioassay did indeed perform better. However, in refid 494, 589, 694 and 769, more positives were detected by PCR. This remains unexplained for refids 589 and 769. In refid 694 preferential isolation of *T. gondii* type II by mouse bioassay is suggested. In refid 494 PCR performs better but the pepsin-digest for mouse bioassay were stored at -4°C before inoculation into mice, which is likely to have killed parasites.

Table 18: Details from publications with mouse bioassay and PCR results. The method performing best is printed bold

Refid		sample	sample size	sample preparation	repeats	Detection method	Positives	Comments
42	PCR	pool of skeletal and cardiac muscles, cerebrum/cerebellum, retina, spleen, liver, uterus, vagina, ovaries and placenta	NS*	Sambrook and Russell (2001)	NS	B1 PCR	3/12	mouse bioassay according to Dubey, 1998, PCR according to Fuentes, 1996, not clear whether nested or conventional was used
	MBio	pool of skeletal and cardiac muscles, cerebrum/cerebellum, retina, spleen, liver, uterus, vagina, ovaries and placenta	NS	pepsin	5 mice	IFAT and microscopy	5/12	
255	PCR	brain	according to manufacturer (25mg)	Dneasy blood and Tissue kit	NS	18S cPCR	2/14 MBio+	methods and results not described very clearly
	MBio	brain	50g	macerated	2 mice	clinical with microscopy/PCR on peritoneal fluid or IFAT and microscopy/PCR on brain	14 (/389)	
494	**PCR**	pepsin digest of 50g muscle	NS	commercial kit	3	commercial qPCR	75/416	aliquot of pepsin-digest for bioassay was stored at −4°C until real-time PCR and ELISA were done
	MBio	muscle	50g	pepsin	5 mice	clinical, microscopy peritoneal exudate and brain	1/14PCR+	
523	PCR	brain and tongue	100mg	Easy DNA kit	NS	529 RE cPCR	2/20 (brain) and 0/20 (tongue)	based on microscopy on mice 5/20, based on PCR on mice 11/20
	MBio	brain, tongue	40g each	pepsin	3 mice	histopathology, IHC, PCR	11/20 (brain) and 9/20 (tongue)	

Refid		sample	sample size	sample preparation	repeats	Detection method	Positives	Comments
555	PCR	pepsin digest of brain, heart, m. gastrocnemius, m. longissimus dorsi	85µg	QIAamp DNA mini kit	NS	529 RE qPCR	22/32	1ml/mouse so 5ml vs 85µg
	MBio	brain, heart, m. gastrocnemius, m. longissimus dorsi	100g each	pepsin	5 mice	serology , microscopy and qPCR	26/32	
589	**PCR**	brain, lung and muscle	manufacturer	spin column kit	NS	529 RE cPCR	22/66 sero+	methods and results not described very clearly, e.g. DNA extraction from homogenate/pepsin-digest or from small tissue sample and tissue matched PCR vs bioassay results not available
	MBio	brain, lung and muscle	25g each	pepsin	4 mice per tissue	serology, microscopy,	20/66 sero+	
625	**PCR**	brain, heart, tenderloin, abdominal muscles	~20g brain, 100-200g other tissues	sequence-based magnetic capture	NS	529 RE qPCR	5/12	
	MBio	brain, heart, tenderloin, abdominal muscles	100g each	pepsin	5 mice	serology and PCR	5/12	
626	PCR	loin and leg	1g	phenol-chloroform	NS	B1 snPCR	0/48	from retail; 3 primers in one tube, 2 times 30 cycles first 65 then 55 annealing temp
	MBio	loin and leg	50g	pepsin	1 mouse	mortality, serology, microscopy	1/48	
694	**PCR**	pepsin-digests of tissue	manufacturer	Dneasy kit	NS	SAG1, SAG2, SAG3, BTUB, GRA6 PCR-RFLP	20/20	preferential selection of genotype II in mousebioassay: 6 mice had pure type II, while 3 of these chickens had mixed infections, 11 PCR positive chickens that failed in mouse bioassay were: 6 type I, 2 type II, 2 mixed I/II, 1 type III
	MBio	brain and heart	NS	pepsin	4 mice per tissue	serology, microscopy, PCR	9/20	
767	PCR	brain, heart, tongue diaphragm and masseter indiidually	15g each	phenol-chloroform	3	529 RE cPCR	25/150	results are on sample level, on pig level all 10 pigs were positive by both methods
	MBio	brain, pool of heart, tongue diaphragm and masseter	50g each	pepsin	5 mice per digest	mortality, serology, microscopy	54/98	

Refid		sample	sample size	sample preparation	repeats	Detection method	Positives	Comments
769	**PCR**	heart	Aspinall, 2002: 1g	Aspinall, 2002: phenol-chloroform	NS	SAG2 nPCR	21/22	the ones with the strongest bands (highest [DNA]) were positive in MBio
	MBio	heart	half of the heart (chickens)	pepsin	not specified	morbidity, mortality, serology, microscopy	11/28 (8/22)	
883	PCR	digests	250µl	phenol-chloroform	NS	B1 cPCR	15/39	pepsin-brain: 23/39 (MBio) and 2/39 (PCR), trypsin-brain: 27/39 (MBio) and 7/39 (PCR), pepsin-diaphragm: 26/39 (MBio) and 3/39 (PCR), trypsin-diaphragm: 21/39 (MBio) and 9/39 (PCR)
	MBio	brain, diaphragm	20g each	pepsin or trypsin (comparison)	4 mice per digest	serology, microscopy	34/39	
919	PCR	brain, heart, m. psoas and m. gracilis	2g	Wastling 1993: prot K digestion followed by boiling	NS	B1 cPCR	7/16	**Results are very close** (no statistical analysis presented): identical for sheep, but one calf appears positive in MBio (only by serology on one mouse inoculated with brain tissue), although in abstract and discussion the authors mention that none of the cattle tested positive; DNA isolation is not optimal (no clean-up), small sample for MBio
	MBio	brain, heart, m. psoas and m. gracilis	1-3g	trypsin	3 mice per tissue	serology, microscopy,	8/16	
950	PCR	placental cotelydon and (in abortions) fetal brain, lung and liver	1cm^3	prot K lysis, boiling, centrifugation	NS	B1 nPCR	23/39	**Results are very close** (not significantly different), MBio performs slightly better on fetal tissues and PCR on placenta: brain 3/6 (MBio) 3/7 (PCR), lung 3/6 (MBio) 2/7 (PCR), liver 4/5 (MBio) 2/6 (PCR), placental cotyledons 13/18 (MBio) 16/19 (PCR); methods are not performed optimally (id. 919)
	MBio	placental cotelydon and (in abortions) fetal brain, lung and liver	1cm^3	no, homogenised	2 mice per tissue	serology	23/35	
1409	**PCR**	lymph	NS	Wastling 1993: lysis of RBC followed by prot K digestion	NS	B1 nPCR	29/97	7 sheep sampled repeatedly, **results are very close** (no statistical analysis presented), 15 discordants with 7x MBio positive and 8x PCR positive
	MBio	lymph	250µl	no, homogenised	3 mice	morbidity, microscopy	28/97	

Refid		sample	sample size	sample preparation	repeats	Detection method	Positives	Comments
1415	PCR	mucosa+submucosa of jejenum, external muscle and serosa of jejunum, tongue, heart, mesenterial lnn., diaphragm	NS	NS	NS	529 RE cPCR	1/4	methods and results not very clear, 1 pig was positive in PCR on mucosa and submucosa, 14 out 62 mice were positive (tissues not specified) leading to the conclusion that 2 pigs were positive.
	MBio	tongue, heart, mesenterial lnn, diaphragm	20g each	pepsin	4 mice	serology	2/4	

* NS = not specified

8.7. Conclusions

Mouse bioassay and PCR are the most commonly used methods for direct detection of *T. gondii* in livestock.

Evaluation using spiked samples is mainly performed for PCR-based assays and often limited to testing of DNA dilution series. Based on DNA dilution series detection limits ranging from 1fg to 100pg have been reported and most methods were able to detect the equivalent of one parasite (85fg). Results with DNA dilution series provide little information about the performance of PCR on samples from infected animals. For that purpose, spiking samples with parasites or tissue cysts prior to DNA isolation would be more informative, but these type of studies are limited. The overview of results with spiked samples (section 8.4) also shows that studies that directly compare different types of detection methods (e.g. PCR in comparison to mouse or cat bioassay) using samples spiked with tissue cysts (with a quantified amount of bradyzoites, e.g. by qPCR) do not exist. These types of studies would be of great value.

From studies that present matched results with two or more direct detection methods for experimentally or naturally infected animals, it is clear that cat bioassay performs best, followed by mouse bioassay and PCR. Detection based on microscopy lacks sensitivity. A more detailed examination of the studies in which mouse bioassay outperformed cat bioassay and of the studies comparing mouse bioassay and PCR appears to confirm that a large sample is the most important determinant of the sensitivity of the direct detection method. Mouse bioassay can be as sensitive as cat bioassay, and PCR can be as sensitive as mouse bioassay when large samples are tested.

In conclusion, for sensitive detection of *T. gondii* cat bioassay, mouse bioassay or a PCR-based method that allows processing of large samples (e.g. by processing many replicates or by performing DNA isolation after artificial digestion or using sequence-based magnetic capture) should be used. It is desirable to avoid animal experimentation; however cat and mouse bioassay demonstrate the infectivity of detected parasites whereas PCR does not. To limit the use of mice or cats, animals are often screened using an indirect test and only seropositives are selected for bioassay. This strategy is useful only when there is a good correlation between indirect and direct detection (evaluated in chapter 9). Another option would be to first screen using sensitive PCR-based detection and select only PCR positives to determine infectivity using cat or mouse bioassay. This strategy was used in reference 494 and this publication also demonstrates the difficulty with this approach: in this case PCR was more sensitive than mouse bioassay, but this was likely due to loss of viability during storage of the pepsin-digest until PCR results were available. For PCR screening followed by bioassay to work, rapid and sensitive PCR-based methods are needed, but the need to test large samples usually increases processing time. Development of sensitive viability assays that are not based on the use of experimental animals would be valuable, but for these type of assays (e.g. tissue culture based) very limited information was available and their performance could not be properly evaluated.

9. Relationship between detection of antibodies and presence of infectious *T. gondii* tissue cysts in meat and other edible tissues

9.1. Introduction

After initial infection with *T. gondii*, the parasite will multiply and spread throughout the body as intracellular tachyzoites. This will trigger an immune response in the host. Although cellular immunity appears to be most important to control parasite multiplication, an antibody response will become detectable within two to three weeks post infection. Under the influence of the host immune response *T. gondii* tachyzoites will tranform into slowly-dividing bradyzoites and form tissue cysts. Formation of tissue cysts protects the parasite from recognition and clearance by the host immune system and *T. gondii* tissue cysts are assumed to persist for life in most intermediate hosts. Nonetheless, the persisting parasites appear to stimulate the host’s immune response to such a level that antibodies remain detectable. When tissue cysts and antibodies persist, a strong correlation between antibody detection and presence of tissue cysts can be expected. Only in the first weeks after infection, parasites are present without detectable levels of antibodies, with IgM appearing first and IgG antibodies appearing later. In that case, direct detection can be positive for seronegatives, especially when a technique is used that can not differentiate between tachyzoites and bradyzoites (e.g. PCR based detection). In reality, demonstrating the presence of parasites in seropositives often fails. Tissue cysts are sparse and concentrations of one cyst per 50g of tissue have been suggested. In addition, their distribution is not homogenuous as some tissues are more likely to be infected than others, and tissue cysts have been observed in close vicinity of each other. Therefore, depending on the direct detection method used and especially on the starting size of the sample tested, tissue cysts can easily be missed. For that reason, a negative result from a direct detection method does not exclude the presence of tissue cysts in the animal that was tested. This is a sensitivity issue of direct detection that is not easily overcome. In addition, discordant results may also result from other sensitivity issues (e.g. autolysis of samples) or specifity issues with the direct detection method (e.g. PCR contamination or false identification of *T. gondii* by microscopy), or sensitivity and specificity issues with the indirect detection method leading to misclassification of animals as seropositive or seronegative.

If a reasonable correlation exists, this means that serological assays can be used to get an indication of the presence of tissue cysts. Detection of antibodies can generally be performed much faster and at lower costs than direct detection of parasites. In addition, serology can be performed on live animals.

This part of the literature review focuses on objective 3: to provide information 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. Therefore, available information on the correlation between detection of antibodies and the presence of *T. gondii* tissue cysts in the main livestock species is collected and summarized. A better understanding of the correlation can direct testing in future, and helps interpreting the public health relevance of the seroprevalence data already available.

9.2. General method

It was decided to focus on the studies that present information that is most relevant for public health, therefore limiting to studies with results using any method for antibody detection in combination with mouse bioassay, cat bioassay or PCR on relevant tissues of naturally infected livestock animals. Mouse bioassay and cat bioassay provide information on infectivity, whereas PCR does not. However, since PCR can give valuable information on presence and many studies use PCR, it was decided to also collect this information. Within a publication, the direct and indirect results had to be presented matched on the level of the individual animal, to be eligible for inclusion in the tables.

The database with relevant records identified in the literature review was filtered for studies on naturally infected animals that reported matched results using a direct and an indirect detection method. These studies were collected separately by animal species. Records were separated into additional entries when more than one direct or indirect detection method was used, or when other sampling or methodological differences required separate collection of data (e.g. differences in sample size or tissue). The database was scanned per animal species and entries that reported direct detection for non-relevant tissues only (e.g. blood, milk, fetal tissues), or used a direct detection method other than cat bioassay, mouse bioassay or PCR were excluded. All remaining entries are included in species-specific tables that report: the direct detection method and sample, the indirect detection method with cut-off value, recovery by direct detection in seropositives, recovery by direct detection in seronegatives, and a comments' field. In these tables, entries that describe a recovery rate of less than 50% in seropositives or more than 5% recovery in seronegatives are highlighted. For entries with highlighted results were the original publications re-examined for methodological issues that might explain the unexpected recovery rate and conclusions from this re-examination are included in the tables as a comment. These 5% and 50% recovery rates were chosen to reflect an acceptable level of discordancy to cover recent infection in seronegatives and the high probability of missing tissue cysts in seropositives (as explained in section 9.1). Note that these rates are only used to identify studies that need a more detailed examination, but are not used to exclude studies from calculation of the overall direct detection rates. The species-specific tables provide an indication of the level of discordancy that remains unexplained after taking into account study-specific methodological issues. Results are discussed by species and an overview of the direct detection rates in seronegative and seropositive animals is provided and discussed at the end of this chapter (section 9.8).

9.3. Relationship in pigs

For pigs 18 entries from 13 publications were included in Table 19. The detection by a direct method in seropositives generally met our chosen recovery rate of 50% (11 entries), and overall the presence of *T. gondii* was demonstrated in 348 (58.8%) of 592 seropositive pigs. The presence of *T. gondii* was demonstrated in 32 (4.9%) of 650 seronegative pigs, and five entries report a recovery rate >5% from seronegatives (out of 13 entries that present results for seronegatives). In 3 entries (757B, 757C and 1291A) this is ascribed to a sensitivity problem of the indirect detection method, i.e. false seropositives. In entry 491 one out three seronegatives tested positive by mouse bioassay, but since only three seronegatives were tested the recovery rate from this entry is uncertain. In addition, these were pigs from farms with a high incidence of *T. gondii* infection, which may mean a high number of animals with a recent infection and therefore a correct discordant result. In 859B the two seronegatives that were positive in cat bioassay, were seronegative in three serological assays (modified agglutination test (MAT), Sabin-Feldman Dye test (SFDT) and Western blot) and the use of SFDT also makes recent infection less likely as SFDT and Western blot also detect IgM antibodies, which rise to detectable levels earlier than IgG antibodies. Direct detection was performed by cat bioassay using 100 to 250g of pooled heart and tongue, which is a very sensitive method. Detection of infection in the cats was by fecal flotation and recovered oocysts were sporulated and fed to mice. The mice were examined microscopically and tested for antibodies using MAT. The specificity of the direct detection method, especially by these experienced authors, is unlikely to be an issue. Therefore, although recent infection cannot be ruled out completely based on SFDT alone, these results suggest that it is also possible to find tissue cysts in chronically infected pigs even though there is a lack of detectable antibodies.

Table 19: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* infection in pigs

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
492	MBio	50g heart	MAT and Safepath ELISA	MAT 1:25, ELISA >0.2	16/30 (0.53)	**1/3 (0.33)**	pigs from two organic farms with high incidence
498	PCR	?g heart	IFAT	1:64	21/38 (0.55)	ND*	
757A	CBio	100-250g heart and tongue	serum Safepath ELISA	>0.106	21/23 (0.91)	0/2 (0.00)	there are also data for semi-nested PCR and qPCR but they have only 4 and 5 positives, as CBio is considered superior for finding tissue cysts, the results with the other direct detection methods are not included in this table.
757B	CBio	100-250g heart and tongue	tissue fluid Safepath ELISA	>0.070	18/21 (0.86)	**3/4 (0.75)**	likely to be problem with the sensitivity of the tissue fluid ELISA, as these 4 were positive in serum ELISA (3 CBio positive), and 3 were also MAT positive (all 3 CBio positive)
757C	CBio	100-250g heart and tongue	MAT	1:20	17/17 (1.00)	**4/8 (0.50)**	sensitivity problem of MAT, as 6 out of 8 were positive in serum ELISA (4 of which were CBio positive)
763	MBio	50g brain and/or heart	MAT	1:20	**15/37 (0.41)**	ND	50g, two mice
769	MBio	10g tissue (heart and/or tongue)	ELISA tissue fluid	not provided	NA**	0/72 (0.00)	poor quality study
793	MBio	50g of pooled heart, brain and tongue	MAT	1:25	**7/28 (0.25)**	ND	no association between antibody titer and isolation, low recovery rate not well explained (50g in 5 mice)
803A	CBio	100g heart	MAT	1:25	60/71 (0.85)	10/203 (0.05)	for seronegatives, first a pool of 5 (100g heart per pig) was fed to cats and if the cat shed oocysts, the pigs were re-fed to cats individually; serum and tissue fluids were tested, but the results appear to be based on the results with serum
803B	CBio	100g heart	Safepath ELISA	0.292-0.321	62/66 (0.94)	8/208 (0.04)	for seronegatives, first a pool of 5 was fed to cats and if the cat shed oocysts, the pigs were tested individually; serum and tissue fluids were tested, but the results appear to be based on the results with serum
859A	CBio	50-100g heart	MAT	1:10	30/30 (1.00)	NA	lot 1, all had titers >=1:100

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
859B	CBio	100-250g pooled heart and tongue	MAT	1:10	19/19 (1.00)	**2/6 (0.33)**	lot 2, additional Western Blot (WB) was done (one additional neg), and SFDT was done for a selection of sera (one additional neg, also neg in WB); no explanation for high recovery in seronegatives, these two pigs were negative in dye test, MAT and WB, recent infection less likely as dye test also detects IgM and WB detects IgG, IgA and IgM
1030	MBio	30g diaphragm	IFAT	1:16	**12/69 (0.17)**	2/40 (0.05)	In the text there are 2/40 seronegatives in the table there are 3 pigs with 1:16 that have a positive MMBio. 30g in 2 mice. Detection in mice by microsopy for mice that died or IFAT for mice that survived for 28 days (not optimal?)
1224	MBio	?g*** diaphragm	IHAT	1:32	**1/12 (0.08)**	0/5 (0.00)	brain and lungs also tested for some of the animals (always negative) but these results can't be matched by animal, therefore only diaphragm results are considered; detection of infection in mice not optimal?: 4-5 weeks, death mice examined microscopically (peritoneal exudate and brain crush smear), half of the survivors bled and serum tested pooled using IHA, in case of positive serology remaining mice were bioassayed in fresh mice.
1233	MBio	55-110g diaphragm	SFDT	1:4	20/33 (0.61)	0/30 (0.00) (0/5 and 0/5 pools)	seronegatives: 25 were tested in pools of 5 and 5 were tested individually
1291 A	MBio	?g diaphragm (entire diaphragm was collected)	IHA	1:4	**8/25 (0.32)**	**1/6 (0.17)**	suitable cut-off could not be established (1:4 and 1:64 is presented)
1291 B	MBio	?g diaphragm (entire diaphragm was collected)	SFDT	1:16	8/8 (1.00)	1/23 (0.04)	
1521	MBio	50g diaphragm	SFDT	1:10	**13/65 (0.20)**	0/40 (0.0)	detection in mice by SFDT 4 weeks pi, seropositives subpassage of brain in mice, SFDT and microscopy (brain) for subpassage mice.

*ND: not determined
**NA: not available
***?g: sample weight not reported

9.4. Relationship in cattle

Data for cattle are limited to 4 entries from 3 publications. Recovery rates in seropositives are low in all entries and in total *T. gondii* was demonstrated in only 4 (3.6%) out of 111 seropositive cattle (Table 20). This is comparable to the results in seronegative cattle, as *T. gondii* was demonstrated in 11 (2.4%) out of 457 seronegative cattle. Note that all direct detection was done using PCR-based methods, therefore not excluding the detection of nonviable parasites.

Record 136 (de Macedo et al., 2012) was excluded from Table 20 as direct detection by mouse bioassay was limited to cow blood and fetal tissues, which are considered non-relevant in this study. The results however are remarkable: Mouse bioassay on cow's blood was positive for 3/29 (0.10) IFAT positive cows and 3/31 (0.10) seronegative cows, and mouse bioassay on fetal tissues was positive for 5/29 (0.17) IFAT positive cows and 9/31 (0.29) IFAT negative cows. Again, similar results in seronegatives and seropositives, but the recovery rates are higher than in the studies included in the table, and these results are based on bioassay.

Table 20: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* infection in cattle

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
429	PCR	0.2-0.3ml masseter	P30-ELISA	Avneg+3SD	**2/73 (0.03)**	7/277 (0.03)	cows, heifers, bulls, calves combined; small sample for DNA isolation (0.2-0.3ml)
579A	MC-PCR	100g heart	ELISA	0.126 at 1:2200 and 0.540 at 1:100	**0/15 (0.00)**	2/83 (0.02)	only if 1:100 and 1:2200 dilution were pos considered ID+, 2 with aspecific binding excluded; lack of correlation for biological reasons is suggested
579B	MC-PCR	100g heart	MAT	1:40	**0/3 (0.00)**	2/97 (0.02)	See 579A
716	PCR	25-50µg heart	IFAT	1:25	**2/20 (0.10)**	ND*	IHC was negative, 25-50µg of heart. Seropositive is based on IFAT, both PCR positives had IFAT titer of 1:100, one was MAT negative and one had a titer of 1:25; 25-50µg of heart for DNA isolation

*ND: not determined

9.5. Relationship in small ruminants

Eighteen entries from 14 publications concerning sheep are included in Table 21. The results clearly demonstrate that a negative result in serology is predictive in sheep: The recovery rate in seronegatives is below 5% in all 11 entries that report direct detection results for seronegatives and overall *T. gondii* was demonstrated in only 17 (1.8%) of 922 seronegatives tested. The overall recovery from seropositives is low (395 (39.4%) of 1002 seropositives) and varies between entries. The recovery rate is below 50% in 10 out of 18 entries, including 2 that had a recovery rate below 10%. In the 2 entries with a recovery below 10% (1176 and 1315) the mouse bioassay protocol is not optimal. However, in 5 out of 8 records with a recovery rate between 10 and 50% no clear methodological issues could be identified. In record 429 only a small sample was tested, and in 883A the sensitivity of the PCR is the issue. In 528A the results apply to the same sheep as 528B but the correlation is determined for MAT (528B) rather than ELISA (528A), the lack of correlation could thus be considered a result of misclassification by MAT. For 589A, 589B, 713 and 736 there is no explanation given or apparent from the materials and methods. For record 679 the authors suggest dilution by pooling with non-predilection sites (brain, heart and diaphragm), however these are all preferential sites according to our analysis of the anatomical distribution in sheep (section 7.5). Although there are 8 entries that have a high recovery rate from seropositives, the fact that the low recovery rate for 5 entries (out of 10) cannot be explained by methodological issues suggests that there may be a biological reason, e.g. low cyst density.

With demonstration of *T. gondii* in 53 (34.9%) of 152 seropositives and 1 (2.0%) of 50 seronegatives (Table 22), the results in goats are strikingly similar to those in sheep. However, with only four entries based on four publications much less information is available.

Table 21: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* infection in sheep

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
429	PCR	0.2-0.3ml masseter, brain	P30-ELISA	not provided	**8/79 (0.10)**	1/71 (0.01)	0.2-0.3ml of brain is used for DNA isolation
457	qPCR	1g brain and masseter	ELISA and IFAT	PP>=20 (ELISA), 1:40 IFAT	11/18 (0.61)	0/78 (0.00)	these are the data for naturally infected sheep only
528A	MBio	120-180g (whole) heart	ELISA	1:4, diaphragm fluid	37/69 (0.54)	9/295 (0.03)	results can also be separated for lambs and adult, available by titer; recovery is higher in adults than in lambs
528B	MBio	120-180g (whole) heart	MAT	1:4, cardiac fuid	**43/97 (0.44)**	3/267 (0.01)	results can also be separated for lambs and adult, available by titer; recovery is higher in adults than lambs
589A	PCR	?g brain, lung, pool of heart and diaphragm	MAT and IFAT	1:16 (MAT and IFAT)	**22/66 (0.33)**	ND*	it is not clear whether seropositive means positive in MAT and IFAT or in either of the tests; low recovery rate is not explained, probably a small sample was tested as DNA isolation was performed using spin-columns according to manufacturer.
589B	MBio	25g brain, 25g lung, 25g pool of heart and diaphragm	MAT and IFAT	1:16 (MAT and IFAT)	**20/66 (0.30)**	not reported/22	it is not clear whether seropositive means positive in MAT and IFAT or in either of the tests, it is mentioned that 22 seronegatives were bioassayed but the results are not reported; low recovery rate is not explained
625	MC-PCR	90-100g heart	ELISA	not provided	26/32 (0.81)	1/23 (0.04)	doubtful ELISA: 7/18
679	MBio	50g pooled brain, heart and diaphragm	MAT	1:25	**16/82 (0.20)**	ND	titers presented in Table, recovery higher with higher titers, mice were considered positive when *T. gondii* parasites were found (not based on serology alone); suggestions: lower recovery by dilution of predilection site with other tissues.
708A	MBio	5-10g ground heart (n=50) or 50g heart (n=17)	MAT	1:25	34/67 (0.51) (18/50 and 16/17)	ND	For 50 that were also tested by CBio: fat, auricles and blood was removed from the heart, myocardium was chopped and ground in blender. Ground myocardium fed to cat and 5-10g that remained in blender was used for mouse bioassay.

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
708B	CBio	~500g (whole) heart	MAT	1:25	35/51 (0.69)	0/44 (0.00) (0/4 pools)	44 seronegative lambs in 4 batches (20-50g of myocardium from each lamb) fed to four cats
713	MBio	50g heart	MAT	1:20	**8/30 (0.27)**	ND	no explanation for low recovery provided. Also no indications (50g myocardium of ewes, 3-5mice, and cut-off of >=1:20)
736	MBio	?g brain	LAT	1:8	**3/12 (0.25)**	1/28 (0.04)	no explanation for low recovery provided, bioassay protocol appears fine, size of tissue sample not provided, LAT not optimal?
883A	MBio, pepsin and trypsin	20g brain and 20g diaphragm	IFAT	1:16	34/39 (0.87)	0/6 (0.00)	separate MBio with pepsin-brain (23/39), trypsin-brain (27/39), pepsin-diaphragm (26/39) and trypsin-diaphragm (21/39) are combined.
883B	PCR	brain and diaphragm digests	IFAT	1:16	**15/39 (0.38)**	0/6 (0.00)	sensitivity of the PCR method is the issue as MBio (883A) gives good correlation
1101	MBio	heart, tongue, limb muscle, intercostal muscle (100g each)	MAT	1:16	8/8 (1.00)	NA**	tissues were tested individually only the data for the slaughter lambs are collected in the table (aborted lambs also presented), they all had high titers (>=1:1024)
1176	MBio	?g brain	LAT	1:2	**5/66 (0.08)**	ND	no explanation provided; no digestion prior to inoculation in mice, mice tested by LAST and microscopy which is possibly not optimal.
1315	MBio	50-80g heart and 50-80g brain	SFDT	1:4	**3/65 (0.05)**	0/46 (0.00)	No digestion prior to mouse inoculation, only microscopy to detect infection in mice.
1518	MBio	100g diaphragm	SFDT	1:16	67/116 (0.58)	2/58 (0.03)	the 2 seronegative MBio+ sheep were old ewes with DT titers >= 1:16 one year earlier

*ND: not determined
**NA: not available

Table 22: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* in goats

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
80	MBio	20g cephalic muscle	LAT	>=1:64, <1:16	13/18 (0.72)	0/4 (0.0)	and 1 DD+/Iddoubt (1:32)
556	MBio	50g heart	MAT	1:5	**28/66 (0.42)**	1/46 (0.02)	results by titer, not serum but clots or fluid from heart; recovery better at higher titers (1/9 1:10, 1/3 1:40, 26/40 >=1:160), better correlation if 1:40 is used as cut-off
666	MBio	50g pool of brain and heart, 50g pool of diaphragm and masseter	MAT	1:25	**12/46 (0.26)**	ND*	titers and pools presented in a table; no explanation provided, but recovery is better at higher antibody titers and better from pools of brain and heart than pools of diaphragm and masseter,
1176	MBio	?g brain (homogenised only)	LAT	1:2	**0/22 (0.0)**	ND	no explanation for low recovery rate provided, but MBio is not optimal: brain suspension inoculated ip in mice, mice positive by LAT were examined by microscopy on brain

*ND: not determined

9.6. Relationship in chickens and turkeys

As chickens are often used to study the worldwide population distribution of *T. gondii* by testing free-range chickens for antibodies against *T. gondii* followed by mouse bioassay on mainly seropositives, many studies reporting some matched results are available. However, whereas seropositives are usually tested individually by mousebioassay, the seronegatives are often pooled and fed to cats. In that case, the results have been separated in different entries in Table 23. This has resulted in 76 entries from 42 publications with many entries limited to seropositives or seronegatives. Recovery by direct detection from seropositives is generally high in chickens, and overall *T. gondii* was demonstrated in 897 (53.4%) of 1679 seropositive chickens (using 4 out of 10 for refid 723A). In 18 out 51 entries reporting on direct detection from seropositives the recovery was less than 50%. In records 448, 723A, 835A and 849A the low recovery rate may have been influenced by storage conditions and autolysis of samples. Entry 795B is a separate entry for chickens with a doubtful titer and recovery from seropositves from that same publication is as expected. For the other publications no clear explanation can be given, but in many cases an increased recovery with titer was observed and generally a low cut-off value of 1:5 in MAT was used. It is interesting to note that in record 513 the recovery was high from the seropositive adult free-range chickens (6/7), whereas none of the 13 seroconverted sentinel chickens was positive by mouse bioassay. The adult chickens had been at the farm for over a year whereas the sentinels had been there for about 70 days, and IFAT titers were higher in the adult chickens. This suggests that time after infection or repeated exposure influences tissue cyst load, and tissues cysts are harder to detect when it is still relatively recent after a primary infection even though a detectable antibody titer has already developed.

As seronegatives are often tested in pools by cat bioassay, it is difficult to get a clear picture of the recovery rate in seronegatives. Excluding refid 1212, out of 2153 seronegatives chickens there were 22 individual chickens that tested positive and between 17 and 353 chickens from 11 positive pools. Therefore recovery from seronegatives can theoretically lie between 1.8% (39 positive chickens) and 17.4% (375 positive chickens), but the upper limit of this range is very unlikely as it would mean that all chickens in the positive pools were positive.

No information was available for turkeys.

Table 23: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* infection in chickens

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
72A	MBio	40g pool of brain, heart and muscle	IHAT	1:16	48/64 (0.70)	ND**	selected for mousebioassay based on IHAT, 42 MAT positives that were negative in IHAT were not tested
72B	MBio	40g pool of brain, heart and muscle	MAT	1:25	48/48 (1.00)	0/16 (0.00)	MAT negatives that were tested were positive in IHAT
129A	nPCR	1g liver	IFAT	1:16	27/29 (0.93)	0/3 (0.00)	
129B	nPCR	1g brain	IFAT	1:16	25/29 (0.86)	0/3 (0.00)	
129C	nPCR	1g heart	IFAT	1:16	16/29 (0.55)	0/3 (0.00)	
266A	nPCR	heart and brain*	IFAT	1:16	15/25 (0.60)	**14/75 (0.19)**	1/2 organ homogenized and max. 1g of homogenate used for DNA isolation. No explanation for detection in seronegatives provided, nested PCR followed by sequencing PCR > increased probability of contamination?
266B	MBio	brain and heart (1/4 of each organ)	IFAT	1:16	8/14 (0.57)	ND	6 isolates, but 8 mice seroconverted
268A	PCR	brain and heart*	MAT	1:5	16/27 (0.59)	0/13 (0.00)	
268B	MBio	brain and heart*	MAT	1:5	**11/27 (0.40)**	ND	complete results are not clearly shown but the authors mention that there is a better agreement when a cut-off of 1:40 is used.
269	MBio	?g pool of brain, heart, spleen, lung, liver and kidney	ELISA	ND	**1/21 (0.05)**	ND	tissue sample not clearly described, authors state that their low recovery rate may be due to the fact that not the whole tissue is inoculated
421	CBio	?g pool of brain, heart and leg and breast muscle	MAT	1:10	7/8 (0.88)	**1/4 (0.25)**	high recovery in seronegatives is not explained, % can be high by chance with only 4 seronegatives tested
448A	MBio	entire heart	MAT	1:5	**1/43 (0.02)**	ND	No explanation for low recovery in ID+ provided but transport of serum and tissue took 10 days (Ethiopia to USA)
448B	CBio	entire heart	MAT	1:5	ND	0/72 (0.00) (0/4 pools)	

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
513	MBio	heart and brain*	IFAT	1:50	**6/20 (0.30)**	ND	none out of 13 sentinel chickens (seroconverted), and 6 out 7 adult free-range chickens; adult chickens on the farm for over a year, sentinel chickens around 70 days, adult chickens also had higher IFAT titers.
563	qPCR	400µl of brain and heart pepsin-digest	MAT	1:25	24/26 (0.92)	ND	separate quantitative results for brain (17/26) and heart (15/26) are presented
616A	MBio	pool of brain and heart*	MAT	1:5	23/40 (0.58)	ND	
616B	CBio	pooled tissues (entire brain and heart?)	MAT	1:5	ND	1-10/10 (1/1 pool)	
676	MBio	pool of heart and brain*	MAT	1:5	**23/81 (0.28)**	ND	titers presented in Table, recovery depends on titer and is especially low at 1:5 (1/26), this will have reduced the overall recovery rate.
694A	MBio and CBio	pool (?) ofbrain and heart*	MAT	1:20	9/20 (0.45)	0/65 (0.00) (0/7-10 pools)	They are using a cut-off of 1:5 for serology, but present the bioassay results with a cut-off of 1:20, therefore 1:20 is the cut-off for this table. The animals with titer 1:5 or 1:10 were tested pooled in mice (3-5 animals), and animals with titers <1:5 were fed to cats in pools of 15 (3 pools).
694B	PCR	?µl of pepsin-digest brain and heart	MAT	1:20	20/20 (1.00)	1-3/20 (1/3 pools)	Regarding seronegatives: only the chickens with titers 1:5 and 1:10 were tested by mousebioassay and will have a pepsin-digest (It is assumed that the 45 chickens <1:5 were not tested by PCR), one pool of 3 animals was found positive There were 11 samples that were PCR positive but did not infect mice, the authors conclude that type II strains were preferentially detected in mouse bioassay.
704	MBio	heart and brain*	MAT	1:5	35/50 (0.70)	0/26 (0.00) (0/5 pools)	5, 8, 5, 6 and 2 chickens in the pools
705	MBio	whole heart, whole brain, 50g of leg muscle separately	MAT	1:40	11/11 (1.00)	NA***	all chickens were seropositive, results are also specified by tissue (heart: 11/11, brain: 5/11, leg muscles: 8/11)

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
723A	MBio	pool heart and brain* (Ghana, Indonesia), not specified (Italy, Vietnam), heart* (Poland)	MAT	1:5	**7/146 (0.05) or 4/10 (0.40)**	ND	in Vietnam no isolation from 80 seropositives, low isolation rates for Ghana, Indonesia and Vietnam possibly due to autolysis of samples; without autolysed samples from those countries 0.40 recovery which is still <0.50, low cut-off of 1:5 resulted in low recovery rate?
723B	CBio	heart* (Poland) or not specified (Indonesia, Italy, Vietnam)	MAT	1:5	ND	1-15/304 (1/10 pools)	seronegatives were tested pooled, 1 pool with 15 chickens from Vietnam was positive, pool in Indonesia consisted of 70 chickens.
723C	CBio	not specified	MAT	1:5	**1/6 (0.17)**	0/14 (0.00) (0/1 pool)	cat bioassay only on samples from Italy, 6 seropositives individually and 14 seronegatives in one pool, low cut-off of 1:5 resulted in low recovery rate? But the one with recovery had a titer of 1:5
736	MBio	pool of brain and heart*	LAT	1:8	**5/20 (0.25)**	**1/5 (0.20)**	no explanation provided, mouse bioassay protocol is standard, misclassification by LAT?
751A	MBio	pool of heart and brain*	MAT	1:10	33/39 (0.85)	ND	
751B	CBio	pool of heart and brain*	MAT	1:10	ND	1-31/45 (1/2 pools)	
754A	MBio	pool of heart and brain*	MAT	1:5	47/66 (0.71)	ND	
754B	CBio	pool of heart and brain*	MAT	1:5	ND	1-8/32 (1/3 pools)	seronegatives tested in three pools, the cat fed tissues from 8 chickens with titer of 1:10 shed oocysts, other two cats were fed chickens with 1:5
756A	MBio	entire heart, ?g pectoral muscles and entire brain	MAT	1:5	**8/19 (0.42)**	ND	increased recovery with titer. Results by tissue: heart (8/19), muscle (3/19), brain (4/19)
756B	CBio	entire heart, 20-25g of pectoral muscles and entire brain	MAT	1:5	ND	0/31 (0.00) (0/4 pools)	
759A	MBio	pool of heart and brain*	MAT	1:5	**22/47 (0.47)**	0/7 (0.00) (0/1 pool)	results per titer presented, recovery increases with titer, seronegatives were tested pooled
759B	CBio	pool of heart and brain*	MAT	1:5	ND	0/16 (0.00) (0/1 pool)	

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
768A	MBio	pool (?) of heart and brain*	MAT	1:5 and 1:20	32/52 (0.60)	0/16 (0.00)	16 with questionable reactions at 1:5 were bioassayed individually in mice (included as seronegatives in this table); seropositives include 30 chickens >=1:5 (batch A) and 22 chickens >=1:20 (batch B)
768B	CBio	pool of heart and brain*	MAT	1:20	ND	0/76 (0.00) (0/3 pools)	3 pools: 48 chickens <1:5 from batch A, 20 chickens <1:5 from batch B, and 8 chickens with 1:5 or 1:10 from batch B
769	MBio	half of the heart	IFAT	1:16	9/15 (0.60)	**2/13 (0.15)**	limited results to the free-range chickens, no explanation for recovery from seronegatives (high rate due to chance?)
778A	MBio	brain*, ?g leg muscle, heart* (pooled or individually)	MAT	1:5	16/19 (0.84)	ND	results from batch 2 (0/20) are excluded because samples were autolysed
778B	CBio	pooled brain*, ?g leg muscle, heart*	MAT	1:5	ND	0/38 (0.00) (0/1 pool)	
779A	MBio	heart and brain*	MAT	1:5	24/33 (0.73)	ND	
779B	CBio	pool of heart and brain*	MAT	1:5	ND	0/17 (0.00) (0/2 pools)	
781A	MBio	pool of brain and heart*	MAT	1:5	**11/36 (0.31)**	ND	There were also 3 chickens with doubtful 1:5 titers and these were pooled and fed to one cat, this cat shed oocysts. Low recovery rate is not explained, low cut-off titer?
781B	CBio	pool brain and heart*	MAT	1:5	ND	0/61 (0.00) (0/2 pools)	
782A	MBio	heart*, ?g pectoral muscles and brain*	MAT	1:10	17/22 (0.77)	ND	
782B	CBio	pool of heart*, ?g pectoral muscles and brain*	MAT	1:10	ND	0/39 (0.00) (0/3 pools)	
783A	MBio	heart*, ?g pectoral muscles, brain* individually or pooled	MAT	1:5	13/16 (0.81)	ND	for >=1:40 (13) tissues were tested individually 15/mice per chicken, for 1:5 (1) and 1:10 (2) tissues were pooled (5 mice/chicken), 1 chicken with 1:10 was positive

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
783B	CBio	entire hearts, brains and 20-25g of pectoral muscle	MAT	1:5	ND	0/30 (0.00) (0/1 pool)	
788A	MBio	pool of heart and brain*	MAT	1:5	23/31 (0.74)	ND	titers are presented
788B	CBio	pool of heart and brain*	MAT	1:5	ND	1-16/32 (1/2 pools)	
791A	MBio	heart*	MAT	1:10, 1:40 and 1:20 depending on batch	**56/218 (0.26)**	ND	no explanation for low recovery rate provided, they mention the low pathogenicity of the isolated strains for mice, with no illness in the mice and only very few tissue cysts in their brains (but they have been tested serologically too, and subpassaged when serologically positive).
791B	CBio	heart*	MAT	1:10	ND	1-122/296 (1/3 pools)	3 pools with negatives (other pools with chickens with unknown titer)
795A	MBio	entire heart, ~20g pectoral muscle, entire brain	MAT	1:20	35/43 (0.81)	ND	individual tissues, 15 mice per chicken
795B	MBio	pooled entire heart, ~20g pectoral muscle, entire brain	MAT	1:5 or 1:10	**1/10 (0.10)**	not applicable	these are the ones with the low MAT-titers, pooled tissues resulting in 5 mice per chicken, recovery is as expected in the >= 1:20
795C	CBio	pooled entire heart, 20-25g pectoral muscle, entire brain	MAT	1:5	ND	0/49 (0.00) (0/4 pools)	
805A	MBio	heart*, ?g pectoral muscle, brain*	MAT	1:20	9/11 (0.82)	ND	
805B	MBio	pooled heart*, ?g pectoral muscle, brain*	MAT	1:5 and 1:10	**1/14 (0.07)**	not applicable	these are the ones with the low MAT-titers, recovery is as expected in the >= 1:20
805C	CBio	pooled heart*, ?g pectoral muscle, brain*	MAT	1:5	ND	0/25 (0.00) (0/1 pool)	
805D	MBio	brain*	MAT	1:20	**1/4 (0.25)**	ND	

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
815A	MBio	brain*, ?g pectoral muscles, heart*	MAT	1:5	10/14 (0.71)	ND	
815B	CBio	pooled brain*, ?g pectoral muscles, heart*	MAT	1:5	ND	0/37 (0.00) (0/2 pools)	
833A	MBio	pooled heart and brain*	MAT	1:10	6/13 (0.46)	ND	
833B	CBio	pooled heart and brain*	MAT	1:10	ND	0/42 (0.00) (0/1 pool)	
835A	MBio	pooled brain and heart*	MAT	1:5	**0/78 (0.00)**	ND	seroprevalence lower than expected, low recovery rate suggested to be due to storage conditions
835B	CBio	pooled brain and heart*	MAT	1:5	ND	5-89/398 (5/22 pools)	
838A	MBio	pooled heart and brain*	MAT	1:5	13/16 (0.81)	ND	
838B	CBio	pooled heart and brain*	MAT	1:5	ND	1-12/24 (1/2 pools)	
842	MBio	pooled heart and brain*	MAT	1:5	**9/19 (0.47)**	ND	no explanation provided
843A	MBio	pooled heart and brain*	MAT	1:5	11/20 (0.55)	ND	
843B	CBio	pooled heart and brain*	MAT	1:5	ND	0/63 (0.00) (0/3 pools)	
843C	CBio	500g total tissue heart, brain, muscle from legs and breast	MAT	1:5	8/9 (0.89)	0/2 (0.00)	one chicken per cat
849A	MBio	pooled brain and heart*	MAT	1:5	**19/49 (0.39)**	ND	results influenced by autolysis of batch 2 (batch 1: 7/9, batch 2: 4/18, batch 3: 8/19)?
849B	CBio	pooled brain and heart*	MAT	1:5	ND	1-15/49 (1/3 pools)	

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
851	MBio	pooled brain and heart*	MAT	1:20	57/69 (0.83)	**4/17 (0.24)**	titers are presented, cut-off is higher (1:20) than in many other chicken studies (1:5), but the four DD+ that were ID- had titers <1:10. Suggestions are: MAT doesn't detect low titers, or recently infected chickens. Overall seroprevalence was high (129/198) so relatively large number of recently infected chickens is not unlikely.
872A	MBio	pooled brain and heart*	MAT	1:40	22/29 (0.76)	ND	higher cut-off than most other studies on chickens
872B	CBio	pool hearts and brains*	MAT	1:40	ND	3-32/52 (3/5 pools)	
1212	MBio	?g of brain and skeletal muscle (homogenisation only)	SFDT	1:2	ND	**27/50 (0.54)**	indirect test is not suitable, as mentioned in the discussion of the paper; ground tissue is inoculated i.p into four mice without prior digestion, mice were examined using non-specific microscopy and SFDT.

* in case of mouse or cat bioassay on heart and brain, the amount of tissue is often not specified, in these cases it can be assumed that the entire organ was used.
**ND: not determined.
***NA: not available.

9.7. Relationship in horses

Very limited information is available on horses as only two studies were eligible for inclusion in Table 23 and one of these studies used pooled testing. The recovery rate from seropositives is low (between 7 (8.8%) and 11 (13.8%) of 80 seropositive horses tested), which is probably only slightly higher than the recovery in seronegatives: Seronegatives have been tested in one study only and they were combined in large pools, therefore the overall number of bioassay-positive seronegative horses lies between 13 (2.4%) and 173 (32.0%) individuals out of 540 tested. This suggests a lack of correlation in horses, similarly to cattle. However, additional studies are needed as only two studies were included and the value of these studies may be limited (see comments in Table 24).

Table 24: Data from publications (identified by Refid) with matched direct and indirect detection of *T. gondii* infection in horses

Refid	Direct method	Sample	Indirect method	Cut-off	Direct detection in seropositives	Direct detection in seronegatives	Comments
255	MBio	~50g brain	IFAT	1:16	**4/46 (0.09)**	10/352 (0.03)	Matched results had to be deduced, and there is some contradiction between text and table.
1227A	CBio	250g-5kg of pooled heart, diaphragm, spinal cord, esophagus per cat	SFDT	1:2	1-5/10 (1/3 pools)	3-163/188 (3/4 pools)	18 pools but 10 cats were fed a mixture of positives and negatives and for one cat the results were not provided so 11 pools were excluded from the table; positive pools (3 pools of 5, 4 and 1 horse) contain far fewer animals than the negative pools (4 seronegative pools: 40, 76, 47 and 25 horses)
1227B	MBio	100g of pooled heart, diaphragm, spinal cord, esophagus	SFDT	1:2	**2/24 (0.08)**	ND*	explanation provided: low recovery may reflect either that the number of *T. gondii* in horse tissue was low and/or that only a small quantity of horse tissues could be inoculated into mice

*ND: not determined

9.8. Conclusions and recommendations

The collected information on direct detection rates in seropositive and seronegative animals is summarized by species in Table 25. Note that the results from a range of methods have been summed up, as classification as positive or negative in both the direct and indirect test is based on the definition in the individual references. The probability of detecting parasites in seropositives was highest in pigs (58.8%) followed chickens (53.4%), sheep and goats (39.4% and 34.9%) and lowest in horses and cattle. Due to pooled testing, the probability of detecting parasites in seronegatives could not be estimated precisely for chickens and horses. In the other species the detection rates in seronegatives were low, but can not be neglected since up to 4.9% was detected in pigs.

This overview shows that there is a lack of information especially for turkeys (no entries), horses (three entries from two records), cattle (4 entries from 3 records) and goats (4 entries from 4 records). In addition, the information available about the detection in seronegatives is less precise than for seropositives as the results with the serological assay are often used to select animals for direct detection. This is especially common when information on the prevalence and distribution of *T. gondii* strains is the main objective and therefore especially true for chickens, as these population studies often make use of backyard chickens and their ability to pick up *T. gondii* from the environment.

Current data suggest that there is some concordance between detection of antibodies to *T. gondii* and direct detection of the parasite in pigs, small ruminants and chickens (Table 25). In pigs, sheep and chickens, recovery rates of up to a 100% (and 72% for goats) have been reported in literature. For these species, in the publications that report a low recovery rate (<50%) in seropositives or high recovery rate (>5%) in seronegatives methodological issues could usually be identified. For example, the recovery from seropositives is generally low when only small tissue sample were tested or when samples for mouse bioassay had degraded before processing. In addition, for these species, many publications report a higher direct detection rate in animals with a higher antibody titer, leading to a higher direct detection rate in seropositives when a high cut-off value is considered and a lower direct detection rate in seronegatives when a low cut-off value is considered and an increased overall concordance when animals with a doubtful serological result are excluded from the analysis. The chosen cut-off value will thus influence the concordance, and, although it is improbable that a single cut-off value can give perfect agreement between direct and indirect detection, it is important to perform studies to obtain suitable cut-off values for indirect tests with reliable estimates of positive and negative predictive value.

The concordance between detection of antibodies and the presence of *T. gondii* in small ruminants, pigs and chickens implies that, in these species, the seroprevalence gives an indication of the risk for consumers. However, it should be noted that absence of antibodies does not guarantee that the meat is free of *T. gondii* and the estimated probability of finding tissue cysts in seronegatives is uncertain due to selective testing of seropositives or pooled testing of seronegatives, and the use of direct detection methods that do not have 100% sensitivity. For small ruminants, pigs and chickens, selection of seropositives remains a sensible strategy in studies focusing on the population structure of *T. gondii*, as it will make more efficient use of the resources needed for genotyping. However, if the correlation between indirect and direct detection is a (secondary) objective, direct detection methods need to be applied equally to seropositives and seronegatives.

The data available on cattle and horses suggest a lack of concordance, with a low overall recovery rate in seropositives and similar rates of direct detection of the parasite in seronegatives and seropositives. The maximum recovery rate reported for seropositive animals was 10% for cattle and 9% for horses. However, it should be noted that limited data are available and, for cattle, all included entries used PCR-based direct detection methods, therefore detection of non-infective parasites is possible. Nonetheless, the same lack of concordance is confirmed in all studies available, and therefore, a biological reason may be more probable than methodological issues. The similar detection rate in seropositives and seronegatives implies that, for these species, detection of antibodies does not give an indication of the public health risk. It also means that selection of seropositives for direct detection is not useful for any type of study, as it is unlikely to increase chances of strain isolation. From a public health perspective, the lack of information on the prevalence of *T. gondii* tissue cysts in horses and cattle is an important data gap: Beef is a major source of meat in many European countries and horse meat in some (e.g. France and Italy) and beef and horse meat are more commonly consumed undercooked or raw than pork or poultry. Based on this overview of the literature, future *T. gondii* prevalence studies in cattle and horses should be based on direct detection (preferably using a method that demonstrates infectivity) and animals should be tested regardless of serological status.

Table 25: 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** Overall[1] (n; 95% CI)	Range[2]	**Detection in seronegatives** Overall[1] (n; 95% CI)	Range[2]	**Kappa-value[3] (95% CI)**	**Interpretation**	**Entries**
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.

10. The relationship between "on-farm risk factors" and *T. gondii* infection

10.1. Quality assessment of the publications

Studies were categorized according to their quality. Categorization was performed by calculating the 0.33- and the 0.66-percentiles of quality scores of all studies. Studies with scores equal or higher than the 0.66-percentile were regarded as "good", those lower or equal than the 0.33-percentile as "poor" and the remaining studies as "average".
Of the 111 studies reported in 75 references 39 were scored "good", 34 scored "average" and 38 scored "poor". In pigs n=35, in sheep n=18, in goats n=3, in studies focusing sheep and goats at the same time n=9, in cattle n=6, in equids n=2 and in chicken n=0 had a good or average quality (Table 26).

In the Tables reporting on risk and protective factors for *T. gondii* infection in farm animals, quality scores are provided and variables from studies with poor quality are indicated.

Table 26: Quality appraisal (WP3) of studies

	Quality of studies			**Total number of studies**
Species	Good	Average	Poor	
Pigs	19	16	12	47
Sheep	9	9	7	25
Goats	0	3	7	10
Sheep and goats	7	2	1	10
Cattle	3	3	3	9
Equids (including horses ponies and mules)	1	1	4	6
Chickens	0	0	4	4
Total number of studies	39	34	38	111

10.2. References and studies included

A total number of 75 references including a total number of 111 individual studies were analysed. References were included once it was assumed that animal husbandry conditions in herds under examination were compatible with European husbandry conditions (e.g. in terms of climate or breed). Of the references finally included n=67 had been conducted in Europe, n=20 in North-America, n=14 in Asia, n=7 in South America and n=3 in Africa (Table 27).

Of all 111 studies, n=106 were cross-sectional studies, n=1 a case-control study and n=2 experimental field studies and n=1 study had a hybrid design. In one case the study type could not be clearly defined.

Only references reporting on cross-sectional studies (n=72) contained more than one study per reference. N=16 references with cross-sectional studies reported on univariable and multivariable data analyses at the same time. Univariable and multivariable studies in a single reference were recorded as separate studies. N=10 references with cross-sectional studies contained more than a single univariable analysis (e.g. separate univariable analyses on different animal species). N=4 references with cross-sectional studies contained more than a single multivariable analysis (e.g. providing more than a single multivariable logistic regression model); these different multivariable analyses resulting in different models were also counted as separate studies.

Table 27: Number of studies included stratified for their origin and the species under examination

	Species							
Continent	Cattle	Chickens	Goats	Equids*	Pigs	Sheep	Sheep and goats	Total number of studies
Africa	0	0	0	1	0	2	0	3
Asia	1	3	1	1	5	1	2	14
Europe	8	0	5	3	27	16	8	67
North America	0	1	2	1	15	1	0	20
South America	0	0	2	0	0	5	0	7
Total number of studies	9	4	10	6	47	25	10	111

* horses, ponies and mules are included

10.3. Information on potential confounders

Many studies reported that differences in *T. gondii* prevalence were associated with the age or the gender of animals, the size of flocks/herds/farms or the geographic location of the flocks/herds/farms. These associations are important; however, variables related to age, gender, flock/herd/farm size and geographic location should not be addressed as “on-farm risk factors” for *T. gondii* infection because most likely they have no direct effect on the risk of infection. Most of these variables should be regarded as confounders or effect modifiers (see age effects).

Age effects: In a total number of 49/58 studies which analysed age effects it was observed that older animals had a higher risk of being positive for *T. gondii*. Only a single study observed the opposite and in 8 of 58 studies no clear age effect was reported. Age effects are attributed to the fact that most of the animals acquire the *T. gondii* infection postnatally and higher prevalence in groups of older animals are explained by a longer time of exposure as compared to the exposure time in younger animals (Table 28). Although variables related to age are important, as the risk of being exposed to *T. gondii* increases with age, this variable can not be regarded as an “on-farm risk factor” because it is related to the individual farm animal and not to the entire farm. In epidemiological study age is optimally included as an effect modifier.

Gender effects: Similar to age, gender is related only to the individual animal, thus also not representing an “on-farm risk factor”. Gender-effects on *T. gondii*-positivity were analysed only in a few studies (9/111). Five of these studies (all conducted in small ruminants) reported that female animals showed a higher risk of being positive. There was only a single study (conducted in pigs) reporting that male animals had an increased risk of being positive and in the remaining studies no clear gender-effect was observed. Experimental studies in mice and guinea pigs have shown a higher susceptibility of females to infection with *T. gondii* (Kittas and Henry, 1979, 1980; Roberts et al., 1995; Roberts et al., 2001) which is in agreement with the findings in epidemiological studies in sheep. Nevertheless, it has to be assumed that other variables, not analysed in these studies but associated with the gender (e.g. differences in age between breeding boars and sows or fattening pigs) and the chance of animals to be detected *T. gondii* positive, have also contributed to this outcome (Table 29).

Table 28: Studies stratified for their outcome on *T. gondii*-positivity with respect to age of the animals analyzed

	Species							
Variable	Cattle	Chickens	Goats	Equids*	Pigs	Sheep	Sheep and goats	Total number of studies
Older animals have a higher risk of being infected/ positive/ diseased	4	1	6	1	22	15	0	49
No clear age effect	1	0	2	1	3	1	0	8
Younger animals have a higher risk of being infected/ positive/ diseased	0	0	1	0	0	0	0	1
Not analysed	4	3	1	4	22	9	10	53
Total number of studies	9	4	10	6	47	25	10	111

* horses, ponies and mules are included

Table 29: Studies stratified for their outcome on *T. gondii*-positivity with respect to gender of the animals analyzed

	Species							
Variable	Cattle	Chickens	Goats	Equids*	Pigs	Sheep	Sheep and goats	Total number of studies
Female higher risk	0	0	1	0	0	4	0	5
Male higher risk	0	0	0	0	1	0	0	1
No clear gender effect	0	0	1	10	0	1	0	3
Not analysed	9	4	8	5	46	20	10	102
Total number of studies	9	4	10	6	47	25	10	111

* horses, ponies and mules are included

Flock/herd/farm size effect: Flock/herd/farm size as a variable to explain differences in positivity for *T. gondii* was analysed in 31/111 studies. In 22/31 studies it was observed that the risk of being *T. gondii* positive was associated with a smaller flock/herd/farm size. Several factors may contribute to these observations and in the following a number of putative reasons are mentioned. A small size of a flock/herd or a farms might in many cases be linked to a lower level of confinement, allowing the introduction of a *T. gondii* infection, e.g. via definitive hosts, other intermediate hosts like rodents and birds or other vectors. In addition, animal feed on small farms is often not prepared in a large scale and not under (semi-)industrial conditions which might facilitate contaminations with *T. gondii*; in addition, on small farms the facilities to store feed might not be adequate and favour contaminations with *T. gondii*. It is likely that many other variables, in addition to those mentioned above and not analysed in most of these studies, are associated with a small size of flock/herd farms and at the same time these variables may increase the chance of animals to be *T. gondii* positive (Table 30).

Table 30: Studies stratified for their outcome on *T. gondii*-positivity with respect to flock/herd/farm size

	Species							
Variable	Cattle	Chickens	Goats	Equids*	Pigs	Sheep	Sheep and goats	Total number of studies
Farm/herd/flock size	0	0	0	0	0	0	0	0
Larger size: higher risk	0	0	0	1	0	2	1	4
No clear size effect	0	0	0	0	2	1	2	5
Smaller size: higher risk	5	1	0	0	11	5	0	22
Not analysed	4	3	10	5	34	17	7	80
Total number of studies	9	4	10	6	47	25	10	111

* horses, ponies and mules are included

Differences related to geographic localization: A number of studies (22/111) reported on geographic differences in the prevalence of *T. gondii* infections in farm animals. Since geographic differences are often associated with a large number of variables having a possible impact on *T. gondii*, including e.g. climatic conditions influencing the survival of *T. gondii* in the environment, or affecting the type of farming, sources of water and fodder for the animals or the prevalence of definitive host in the surroundings of a farm. However, geographic differences do not offer direct information on "on-farm risk-factors" for *T. gondii* and have therefore to be regarded to be confounders (Table 30).

Table 31: Studies stratified for their outcome on *T. gondii*-positivity with respect to the geographic location of flock/herd/farm

	Species							
Variable	Cattle	Chickens	Goats	Equids*	Pigs	Sheep	Sheep and goats	Total number of studies
Geographic region of farm/herd/flock location	4	0	5	2	7	4	0	22
Not analysed	5	4	5	4	40	21	10	89
Total number of studies	9	4	10	6	47	25	10	111

* horses, ponies and mules are included

10.4. On farm risk factors in pigs

There were 32 epidemiological studies in 22 references available providing information on potential "on-farm risk or protective factors" for *T. gondii* infections on pig farms (Appendix E, Supplementary Table S1).

Definitive host related variables: The most often reported risk and protective variables were variables associated with the definitive hosts of *T. gondii*, i.e. domestic cats. Infected cats are known to shed large numbers of environmentally resistant oocysts with their faeces for a short period of time after infection. This may contribute to infection of farm animals via a direct contamination of feed or water or indirect via the infection of other intermediate hosts. Since pigs are omnivorous, other intermediate hosts infected with *T. gondii* could also serve as a source of infection for pigs.

There was only a single reference that demonstrated a direct statistical association of oocysts contaminations with an increased risk of infection in pigs. This reference reported in four different studies that the observation of *T. gondii* oocysts in cat faeces, pig feed or soil was statistically associated with an increased risk of infection in pigs (Refid 1008).

Studies analysed the effect of domestic cats on *T. gondii* in farm animals in different depth: not only the access or the presence of cats were identified as risk factors, but also the possibility to have contact to cat faeces or a high cat density on farm or a high frequency of exposure to cats was associated with an increased risk of *T. gondii* positivity (Appendix E, Supplementary Table S1).

A number of references also reported that with increasing numbers of cats also the risk of positivity in pigs increased (Appendix E, Supplementary Table S1). It is difficult to generalize all these studies because some studies only analysed numbers for specific sub-categories of cats, i.e. juvenile or seropositive cats. The rational for a restriction of an analysis to young cats was based on the assumption that young cats were naïve and non-immune and thus may likely be able to shed oocysts after infection. A restriction to seropositive cats was regarded as a straight-forward approach, because seropositive cats may represent those cats having shed oocysts recently or at a time in the past and thus most likely contributed to the infection on-farm.

Overall these studies clearly show that cats on farms have a key role for the infection of swine. Therefore, measures to prevent shedding of *T. gondii* oocysts by farm cats might be very efficient in reducing the incidence of *T. gondii* infection in pigs. There is a single study reporting that an experimental vaccine administered to cats conferred some protection against *T. gondii* infection in fattening pigs (Refid 914). However, overall the protective effects of this farm cat vaccination on the *T. gondii* prevalence in different swine populations were statistically significant at a single but not at all examination points after intervention and not statistically significant in other pigs than fattening pigs (Refid 914).

Feed-related variables: It is hypothesized that one important route by which pigs become infected on-farm is via the ingestion of feed contaminated by *T. gondii*. These contaminations may most likely represent contaminations by oocysts or by infected intermediate hosts like rodents. It is therefore not surprising that most of the variables characterizing a situation in which feed contamination seems to be possible were associated with risk, while variables characterizing a situation under which contamination is unlikely were protective in most of the analyses (Appendix E, Supplementary Table S1).

In a single study feed storage in a silo was a risk relative to feed storage in a warehouse (Refid 38). This finding is hard to generalize because nearly nothing was reported on the specific situations on the study farms and on more details of food storage in these farms.

In another study (Refid 1380), fluid feeding was found protective while dry feeding posed a risk for *T. gondii* farm positivity. Again it is difficult to explain the reason for these findings. From the biological point of view, fluid feed would provide optimal conditions for a survival of *T. gondii* oocysts (given that the temperature of fluid feed is low). On the other hand, providing feed in a liquid consistency might be associated with a lower risk of secondary contamination of the feed, because fluid feed is provided to the animals via a pipe system most likely not accessible for cats or rodents. Even when equally accessible, cats are more likely to defecate in dry fodder than in fluid feed. However, it is also possible that variables associated with the consistency of feed exert no direct protective or risk effect because fluid feeding of pigs requires specific technical equipment. Therefore, it is likely that the variables on consistency of feed are also associated with the size of the farm or the farm type and are not directly linked to the infection risk of animals, i.e. are confounders.

Housing related variables: There was only a single reference looking on the effects of housing (refid 1380). While the use of straw bedding on farm posed a risk the use of perforated or slatted floor was protective. Although biologically plausible, due to the hypothesis that straw bedding may favour the survival of oocysts or the presence of rodents as intermediate hosts, it has to be taken into consideration that also variables related to floor or housing may represent confounders due to their likely association with the farm type and the farm size.

Variables related to housing-in/out, cleaning and disinfection: It is likely that the intensity of cleaning and the period of time spent for cleaning, the ways by which the animal house is disinfected prior to housing-in new animals are important with respect to the occurrence of oocysts contaminations or rodents as potential sources of infection. Therefore, it is not surprising that the duration of the time period a pig-pen is empty prior to housing-in new animals is associated with protection (Refid 1380).

Farms following the all-in-all-out regimen were found protected while those not following this regimen were at risk of being *T. gondii* seropositive (Refid 1380). A possible explanation for this observation could be that thorough cleaning is favoured once animals are not housed-in continuously but an all-in-all-out regimen is followed. Therefore the association of variables related to all-in-all-out regimens with *T. gondii* positivity is biologically plausible (Appendix E, Supplementary Table S1).

The difference between mechanical or manual cleaning is not understood (Appendix E, Supplementary Table S1). It is likely that variables related to the way of cleaning are confounders because they are most likely associated with the size of the farm and the type of farm. The same seems to be true for variables related to disinfection. The effect of disinfection is not clear because the oocysts stage of *T. gondii*, i.e., the stage most likely responsible for infection is known to be resistant against most of the disinfecting substances on the market and consequently it is not likely that there is a direct effect of disinfection on *T. gondii* although – once the farmer has selected the appropriate substance – an effect can not be ruled out completely. Nevertheless, it can be assumed that farmers using disinfection are more thoroughly cleaning their facilities and that not the disinfection itself but the preceding thorough cleaning is responsible for the protective effect of disinfection related variables.

Variables associated with the level of confinement: A direct or indirect infection via contamination with *T. gondii* oocysts present in the environment of pigs is more likely to occur once pigs are kept outside of a pig-pen or kept on pasture, because pigs could come into closer or a more frequent contact to potentially infected cats and intermediate hosts or to pre-existing oocyst contaminations outside stable. Therefore it is biologically plausible that variables related to the level of confinement have an effect on infection risk and there was a large number of references confirming this (Appendix E, Supplementary Table S1). Herds with outdoor access are at risk and also in most studies pastured swine turned out to be at risk of being seropositive for *T. gondii*.

Rodent-related variables: Pigs are omnivores and may ingest carcasses of rodents often occurring in large numbers on swine farms. Rodents are intermediate hosts of *T. gondii* and pigs may become infected due to the presence of *T. gondii* tissue cysts in these intermediate hosts. Thus, rodents may pose a risk for *T. gondii* infection directly. If cats are present on farm, *T. gondii* rodents as prey for cats may act as risk factor because cats may shed *T. gondii* oocysts after the ingestion of infected prey. There was one study showing that cats used for rodent control was posing a risk (Refid 1380)

Nevertheless, the direction (i.e. increasing risk or protection) in which rodent-related variables may act is not unambiguous in studies analysing rodent related variables (Appendix E, Supplementary Table S1). On one hand farms with no rodent control or the presence of *T. gondii* seropositive rodents were at risk which is expected. On the other hand there are also studies suggesting that the presence of rodents was protective or the use of chemicals, traps or destruction of habitats against rodents posed a risk. It is difficult to find explanations for these conflicting results. It is possible that although rodent control is done the efficiency of these measures is not enough to exert a protective effect.

Variables related to water provided to animals: Water is an ideal medium for the survival of *T. gondii* oocysts. Therefore it is plausible that variables associated with a potential contamination of water with oocysts (Drinking water provided in a trough [Refid 1380]) or providing surface water as drinking water (Refid 404, 578) pose a risk. However, it is also possible that that these variables are confounders and indicators for other more relevant risk factors (i.e. level of confinement).

Biosecurity related: It is likely that biosecurity-related variables are only partially biologically relevant. Variables characterizing a low level of personnel hygiene (e.g. No boots, no overall, no protective clothing available; refid 1380) posed a risk. Although a low level of personnel hygiene could contribute to an accidental contamination with *T. gondii* in the animal house, it is unlikely that a low level of personnel hygiene could contribute to large numbers of infections in pigs. However, variables characterizing a high level of personnel hygiene are likely associated with larger farms or farm types characterized by an intensive swine production. These intensive swine production farms follow per se regimens protecting pigs from *T. gondii* infection, e.g. via a high level of confinement.

While the adequate removal of dead animals was protective the inadequate removal of dead animals posed a risk (Appendix E, Supplementary Table S1). Both could be variables with a biological relevance because dead and eventually *T. gondii*-infected animals could serve as a source of infection for definitive hosts or other intermediate hosts like rodents, contributing to infection of swine. However, again it is also possible that these variables are confounders because they may reflect just farm type or farm size and thus the general hygienic conditions or the level of confinement on farm.

Other variables which characterise a low level of biosecurity like “No insect control”, “No bird-proof nets”, “Presence of mosquitoes and flies” and which were associated with a risk of *T. gondii* positivity (Appendix E, Supplementary Table S1). It is possible that these variables represent confounders which reflect the general hygienic conditions or the level of confinement on farms. However, a mechanical transmission of oocysts via insects might be another plausible explanation for these findings.

Climate related: One reference reported that farms located at regions with higher humidity, more rainfall and higher temperature had a higher risk of seropositivity (Refid 604). This effect of climate related variables could be explained by the fact that a high humidity could favour the survival of oocysts and a higher temperature could shorten the sporulation time of oocysts, i.e., the period of time after which oocysts become infectious.

Season related: There was a single study looking at a season effect on seropositivity of slaughtered fattening pigs and observed in a very limited number of farms that pigs slaughtered in autumn or winter had a higher risk of being positive compared to pigs slaughtered during other seasons (Refid 789). This is in agreement with other studies reporting on seasonal effects regarding the proportion of cats shedding *T. gondii*-oocysts and it is possible indeed that there are seasonal effects on the prevalence of positive pigs on farms.

Related to the extent of specialization: Farms that showed indicators of a low level of specialization (such as backyard farming or other livestock or animal species on farm) had a risk of *T. gondii* positivity (Appendix E, Supplementary Table S1). It is unlikely that the presence of all kind of other livestock species on farms had a direct effect on the risk of pigs being *T. gondii*-positive. Since goats and sheep are highly susceptible to infection it is not unlikely that their presence could have an direct biologically effect, because the presence of highly susceptible animal species like sheep and goats on farm increases the probability that a cyclic transmission of *T. gondii* occurs and that contamination of fodder, drinking water or the environment of pigs with *T. gondii* oocysts or other *T. gondii*-infected intermediate hosts like rodents occur. However, these variables are most likely also indirect indications for the size and the type of farm. Less specialized farms are less likely industrialized and thus might be less well equipped. This might affect the extent to which e.g. contamination of fodder or drinking water on pig farms is possible.

Related to the purpose of livestock: Pigs from farms not belonging to the feeder-to-finish type (e.g. farrow-to-finish, weanling-to-feeder, weanling-to-finish) had an increased risk of being positive (Appendix E, Supplementary Table S1). This is difficult to explain and variables on the farm-type are most likely confounders. It is possible that feeder-to-finish farms are often larger than other farms and therefore these farms need to be optimally managed in terms of biosecurity and hygienic measures. This may contribute to a reduced infection risk for *T. gondii*. In addition, the finding that pigs from feeder-to finish type farms have a lower prevalence than pigs from other farms could be related to the age of the sampled animals. In other farm types older animals might have been sampled than in feeder-to finish farms and since the probability of exposure to *T. gondii* increases with age this could explain effects related to farm type. This could be a particularly relevant explanation because not in all studies the effect of "age" was considered as an important confounding variable during analysis.

Related to potential effects of toxoplasmosis: There are indications that *T. gondii* infection in pigs has adverse effects on the reproduction of swine. There are two references in which the results of epidemiological analyses are in agreement with this hypothesis, suggesting that farms with reproductive problems or an increased mortality in weaning have a higher risk of *T. gondii* infection (Refid 621, 812). However, problems in reproduction or mortality in weaning are putative effects of a *T. gondii* infection. Potential effects of the infection should not be regarded as "on-farm risk factors".

Interactions: Only two studies analysed interactions. In one reference it was observed that pigs from farrow-to-finish farms were at risk once these farms performed no rodent control or in addition cleaned the animal house manually (Refid 404). In the same reference also small farms had a higher risk once located lower than 200 m above sea level (Refid 404). Another study indicated the farms on which are no cats and sows are entirely pastured or kept in partial confinement had a higher risk for *T. gondii* positivity as compared to pastured sows with cats present (Refid 1011). This is an indication that the risk posed by cats is strongly affected by the settings on farm and that the pure presence of cats is not the only variable that affects positivity of swine farms. Nevertheless, presence of cats turned out as a risk factor for *T. gondii* posititivity in the same study (Refid 1011).

10.5. On farm risk factors in cattle

In cattle there were only three epidemiological studies (out of nine included) available providing information on potential "on-farm risk or protective factors" for *T. gondii* infections on cattle farms (Appendix E, Supplementary Table S2).

Definitive host related: In one study conducted in France (Refid 405) the presence of cats turned out as a statistically significant risk factor in a model explaining cattle herd prevalence. This is in accordance with the findings in other animal species (e.g. pigs) and is explained by the role of cats as definitive host in the lifecycle of *T. gondii*. In farms where cats are present it is more likely that fodder or drinking water provided to cattle is contaminated with *T. gondii* oocysts than on farms without cats.

Related to water provided: In the above mentioned study in France (Refid 405), a water point provided on pasture was associated with risk in a model to explain cattle herd prevalence. The reason why a water point on pasture could pose an increased risk for *T. gondii* infections in cattle was not explained in the respective reference. However, it is possible that these water points – under the assumptions that oocysts shed by felids are contaminating the water – provide ideal conditions for the survival of these oocysts and thus also for a prolonged and efficient transmission of *T. gondii*.

Level of confinement-related: Surprisingly in a study from Serbia (Refid 1386) cattle that were kept exclusively in total confinement had a higher risk of being positive than cattle that had access to outside pens. This is in contrast to the observations made for other animal species in other epidemiological studies. The authors of this study could not provide an explanation for this finding and argued that farms providing cattle access to outside pens might differ from farms on which cattle are kept in total confinement in the way feed is stored. In addition, it is possible that at cattle farms with no outside pens cattle had a closer contact to domestic cats and their excretions than at other cattle farms.

Related to cattle density on farm: There was a study from Spain (Refid 463) reporting that cattle from herds with low cattle density had a higher risk of being seropositive than cattle from herds with a higher cattle density. There was no explanation provided in the respective reference and it is most likely that the variable "cattle density" represents a confounding variable associated with other biologically relevant variables (e.g. variables related to the protection of feed and drinking water from contaminations or the presence of cats close to the farm animals).

Related to the geographic localization: In a study from France (Refid 405) the isolation of farms (i.e. a farm has no neighbour farm) explained higher herd prevalences. A conclusive explanation could not be provided in this reference. It was mentioned that farm isolation was statistically significantly associated with farm size and it was suggested that isolated farms might have "a more traditional management", and may differ in feeding practices (e.g. in feeding less often silage).

Related to interactions between variables: In the study reported from France (Refid 405) two interactions were significantly contributing to a model explaining the within herd *T. gondii* prevalence. One was the interaction between Neighbourhood-index and Farm size. The Neighbourhood-index had three levels: isolated, one neighbour, two or more neighbours, i.e. a low, medium and high Neighbourhood-index, respectively. The interaction showed an increased risk especially in smaller farms once they had a lower neighbourhood index (i.e. a higher risk especially in small and isolated farms). Because in this study (Refid 405) isolated farms also correspond to the smallest herds, the authors argued that unknown variables related to management (possibly related to feeding silage) and environmental variables specific for small farms contributed to this effect.

The other interaction (Refid 405) addressed an effect of age once cats were present or absent at farms. Once cats were present, especially younger cattle had a higher risk of being seropositive while older cattle had a lower positivity risk. This unusual effect on farms were cats were present, was explained by assuming a high level of exposure in young cattle (i.e. calves) resulting in a strong immune response which is efficient still in adult cattle. In the absence of cats the age-effect followed the usual pattern with higher prevalence in older animals, which was explained by the higher probability of low level exposure to *T. gondii* in older animals. Further studies are necessary to confirm these findings.

10.6. On farm risk factors in small ruminants

There were 32 epidemiological studies in 20 references available providing information on potential "on-farm risk or protective factors" for *T. gondii* infections on small ruminant farms (Appendix E, Supplementary Table S3).

Definitive host related: Similar to the findings in epidemiological studies on other species many studies on small ruminants provided evidence that variables related to definitive hosts (mainly domestic cats) are important risk factors for *T. gondii* infection. In small ruminants these variables included the presence of cats or young cats on farm, the contact or access of cats to fodder, water or pasture and the contact of farm animals with felines (Appendix E, Supplementary Table S3). With this in mind, a finding of one of the studies was surprising; the absence of wild felines was associated with a risk (Refid 440). A possible reason could be that in the particular region were this observation was made (Ethiopia) the presence of wild felids is associated with a remote location of the farm. A remote farm location might be associated with only small numbers or the absence of domestic cats. Although wild felids also might be able to serve *T. gondii* as definitive host, wild felids might have only a low relative importance because their numbers are usually small and they or their excretions have no close contact to domestic ruminants.

Feed-related: Feeding concentrate (Refid 471) or minerals (Refid 636, 745) to small ruminants was associated with risk and also the type of mineral supplementation (common salt *vs* mineral salt, Refid 745) seems to have an influence. It is unlikely that feeding concentrate or salt may have a direct effect on the risk of *T. gondii* infection. It is more likely that these findings are confounders. Feeding concentrate or minerals could increase the infection risk of small ruminants because of the possibility that these additional feeds become contaminated, e.g. with *T. gondii* oocysts during storage or when provided. Feeding hay posed a risk relative to feeding on pasture or feeding fresh bulk feed (Refid 636). This is surprising because most likely dry hay does not favour the survival of *T. gondii* oocysts. Oocysts survive best under humid and cool conditions. However the storage of hay and the way hay is provided to the animals might include possibilities of a secondary contamination of this type of feed and thus increase the infection risk of animals fed hay. In addition, hay is usually provided to animals kept in stables or close to farm buildings. This might be associated with the possibility of the animals to come into close contact to domestic cats and other intermediate host (e.g. rodents). In a study conducted in Romania (Refid 1386) animals that were fed via a manger, a trough or on pasture had a higher risk of seropositivity compared to those only fed via manger or a trough (but not on pasture).This finding is hard to explain but it suggests that feeding on pasture may have provided an additional risk to become *T. gondii* positive.

Related to water provided to the animals: Water contaminated by *T. gondii* oocysts is regarded as important source of infection. However, in summarizing epidemiological studies addressing these issues in small ruminants, there was no clear answer on which sources of water could pose an increased risk compared to others (Appendix E, Supplementary Table S3). In the various studies, both, tap water as well as surface water were found to pose a risk. In other species, e.g. in swine a risk was identified when surface water was provided to the animals and providing tap water was associated with protection. The reason that there was no unambiguous effect of the source of drinking water in small ruminants may indicate that the effect of the different sources of water had been covered by the effects of other more important risk factors which were associated with the sources of water in some of the studies.

Housing related: In a Norwegian reference (Refid 945) the outcome of studies showed that timber construction of a sheep house provided protection although no explanation for this finding could be provided and the effect of this variable was regarded as a confounder. In the same reference the existence of a perforated metal floor in the sheep house provided a protective effect which was explained by a more efficient removal of litter, including also possible contamination with *T. gondii* by this type of floor. Findings in other species, e.g. swine support this hypothesis because the use of a floor other than grid, full slatted floor, partially slatted floor or the use of straw bedding in the pig-pen posed a risk as shown in a single study (Refid 1380).

Level of confinement and management intensity: The results of studies analysing the effect of different levels of management intensity are conflicting. There are a number of studies comparing flocks with higher levels of management intensity with those of lower management intensity. Summarizing these studies provided no clear picture: a higher as well as a lower management intensity posed a risk in the different studies. This is a clear indication that variables characterizing the level of management intensity are most likely confounding variables. Underlying biological relevant variables were not clearly identified in the respective studies.

Rodent related: In contrast to pigs, small ruminants are not omnivorous. Nevertheless, the presence of rodents or the use of mouse poison (which could be regarded as an indicator for the presence of rodents) were associated with a risk (Refid 745, 945). The presence of *T. gondii*-infected rodents may favour indirectly a transmission of *T. gondii* to small ruminants via oocysts shed by cats predating these rodents.

Biosecurity related: A single study from Scotland (Refid 1622) suggested that limited contact of sheep with animals from other herds confers protection (i.e. no common pasturing with sheep from other herds, farm land having a border only to a single other farm). This could be explained by hypothesizing that farms with more contacts to others more likely come into contact with other potentially infected small ruminants or definitive and intermediate hosts, making an indirect transmission of the infection (e.g. via definitive hosts) more likely.

A study from Jordan (Refid 531) addressed variables to explain *T. gondii*-positivity in abortion. In this study different ways of disposing aborted material were analysed. Interestingly, the habit of feeding foetuses to dogs seemed to confer protection. Because of other reasons the disposal of abortion material via dogs is not appropriate. Nevertheless, this finding suggests the importance of effective measures to prevent the contact of potential definitive and intermediate hosts with aborted material and eventually also afterbirths. This observation is also in accordance with finding in epidemiological studies on other species, e.g. swine.

Although the introduction of animals from other farms has to be regarded as an indicator for a low level of biosecurity, a study from Serbia reported that goats coming from other farms were less likely seropositive than animals born on the farm. This was explained by a lower *T. gondii* seroprevalence in geographic regions were these replacement animals had been purchased.

Climate related: One study observed that animals reared at semi-warm humid climate had a higher prevalence than animals kept in other climates (semi-warm sub-humid, temperate sub-humid). These findings are in agreement with experimental data. Experimental studies showed that survival of oocysts is increased by humidity. Higher temperatures support sporulation and shorten the interval between shedding and the attainment of infectivity of oocysts. However, high temperature is not beneficial for the survival of sporulated oocysts.

Related to the extent of specialization: Variables regarded as potential indicators of the extent to which a farm is specialized are most likely confounders and have no direct effect on a *T. gondii*-infection risk. Indicators for a higher level of specialisation (e.g. no mixed breeds, only milk production, no other livestock species on farm, mixed exploration (dairy and meat) vs only meat exploration) seemed to confer protection (Appendix E, Supplementary Table S3). Higher levels of specialisation are likely associated with better hygienic conditions, higher levels of confinement etc. as already stated for variables regarded as indicators for level of confinement and management intensity. The only variables which may have a biological relevance are those related to the presence of other animal species on farm. Since other species are potential intermediate hosts of *T. gondii* the presence of other, potentially infected animal species could represent sources of infection for definitive hosts of *T. gondii*.

Related to the use of individual animals: The reason why animals used for meat production have a higher risk of being *T. gondii* positive (Refid 993) is not clear. We believe that this variable is a confounder and is potentially related to differences in feeding, pasturing, proximity to sheep barns and thus to exposure to domestic cats and other intermediate hosts.

Related to potential effects of toxoplasmosis: It is not surprising that farm/flock positivity in some studies could be linked to putative *T. gondii* associated effects (Appendix E, Supplementary Table S3), i.e. to events putatively associated with toxoplasmosis (including occurrence of abortion outbreaks, period of gestation at which an abortion occurred, neurological problems in lambs, mortality 24 h after delivery). Although these variables are associated with potential effects of existing *T. gondii* infections on a farm or in a flock these events could potentiate *T. gondii* positivity via a further dispersal of infection.

Potentially farm/flock size related: In one study conducted in British sheep flocks it was observed that the likelihood of seropositivity increased with the number of breeding ewes on a farm (Refid 541). A possible reason is that with increasing flock size the likelihood of a presence of positive animals increased and also the possibility of a dispersal of the infection to other animals in a flock.

Geography related: There is no clear direction of effects the location of a farm above sea level has. A potential reason is that studies are conducted in different areas of the world (Appendix E, Supplementary Table S3). Therefore, a particular level above sea is associated to very different climatic conditions with very different effects on the lifecycle of *T. gondii*.

In studies reported from Spain the proximity of farms to other farms (i.e. < 500 m distance to another farm) turned out to be protective (Refid 993). The explanation given was that proximity to other farms is also associated with a proximity to a village and with public water supply. Public water supply could serve as an explanation for a lower *T. gondii* infection risk.

In a Norwegian study (Refid 945) “black soil” on a farm or a pasture was regarded as a risk factor. There was no explanation provided and also no information on other forms of soil not associated with a risk.

In a study from Ethiopia (Refid 440), both flocks with grazing land only located in a plain area and flocks with pastures located only in mountainous area were at risk as compared to flocks with mixed (i.e. mountainous and plain area) pastures. An explanation for this finding was not provided and it is not unlikely that variables characterizing the texture of pastures are confounders.

Land cover related: A study conducted in Greece (Refid 25) reported that Savannah-like environment of a farm conferred protection relative to forest or urban/crop environment. An explanation was not provided. It can be hypothesized that a Savannah-like environment does not favour survival of oocysts due to micro-climatic condition (low level of humidity and high temperature). In addition, the higher risk in areas with an urban/crop might be attributed to larger numbers of cats or intermediate hosts of *T. gondii* (e.g. rodents, birds, other livestock) being present in those areas.

10.7. On farm risk factors in chickens

Only three studies were available providing information on potential risk and protective factors in chicken (Appendix E, Supplementary Table S4). There was no eligible epidemiological study providing information on “on-farm risk factors” for *T. gondii* in turkey.

Variables related to breed: The reason for broilers having significantly lower seroprevalences than layer chicken remains unexplained. As a possible reason the authors of the respective study from China (Refid 479) suggested that broilers examined in this study had a lower age compared to the age of breeder and layer chicken.

Variables related to the extent of specialization: In a study from Mexico (Refid 518) backyard chicken had a higher risk of being seropositive compared to chicken reared at large farms. Feeding of backyard farmed chicken usually includes practices (i.e., feeding from the ground, feeding waste, limited cleaning and disinfection) which may favor a contamination with *T. gondii* while chicken from large farms are most likely fed with fodder produced under industrial conditions and are reared in well equipped, cleaned and disinfected animal houses or pens.

Variables related to the level of confinement: Free range chickens were shown to have a higher risk of being seropositive compared to chicken reared at large farms (Refid 479, 683). This is in in accordance to the findings in pigs.

10.8. On farm risk factors in equids

In equids only four references provided some information on potential on-farm risk or protective factors for *T. gondii* infections (Appendix E, Supplementary Table S5).

Related to extent of specialization: Similar to the observations in other animal species, a low level of specialization on farm, as indicated by the simultaneous presence of different livestock-species, i.e. the presence of domestic ruminants, is posing a risk for *T. gondii*-positivity (Refid 495). Domestic ruminates, such as sheep and goats, are highly susceptible to *T. gondii*-infection. It is possible that the presence of infected small ruminants on-farm could indirectly contribute to the infection of equids once cyclic transmission of *T. gondii* is completed via domestic cats. However, the presence of other types of livestock on a farm also indicates a traditional farm management which may imply that equids are less likely kept in confinement and that cleaning and disinfection protocols are only basic.

Related to geographic localization: Horses reared in rural areas were shown to have a higher risk of being seropositive compared to horses reared in urban areas (Refid 490). An explanation might be that horses in rural areas are living in environments which are better suited for the propagation and the survival of *T. gondii* due to a larger spectrum of intermediate hosts. Additional reasons might be that horses in rural areas are kept less confined than horses in non-rural areas and are fed with other types of roughage or more likely pastured as compared to horses in an urban environment. Grazing may favour the infection with *T. gondii*.

Related to purpose of livestock: Horses used for agricultural work and equids used for farming had a higher risk of being *T. gondii* positive compared to horses used for shows or equids used for racing. The same reasons may apply as those already mentioned in the previous paragraph. Horses used for agricultural work may live in environments which are be better suited for the propagation and the survival of *T. gondii* and are fed in another way than horses used for shows or racing horses (Refid 622, 1379).

10.9. Summary on relationships between on farm risk factors and *T. gondii* infection

In the following paragraphs those categories of variables which were identified either as risk or as protective factors for *T. gondii* infection in many of the studies on different farm animal species are discussed. The variables identified should not be regarded as definitive, since almost all studies were cross-sectional studies. Associations identified in cross-sectional studies only allow to form hypotheses.

The categories of variables discussed in the following paragraph include only those for which the current knowledge of the parasite provides evidence that these variables are biologically relevant. Biologically relevant variables are related to or associated with the putative routes by which *T. gondii* is transmitted from felids to farm animals (Fig. 3). This includes variables related to the infection of felids as definitive hosts of *T. gondii*, related to the sporulation, survival and dispersion of oocysts and related to the oral ingestion of infectious material by livestock as intermediate hosts of *T. gondii*. However, it has to be kept in mind that also those variables identified only in a single or in a small number of studies, i.e. variables discussed above but not included in this section, could be relevant risk or protective factors.

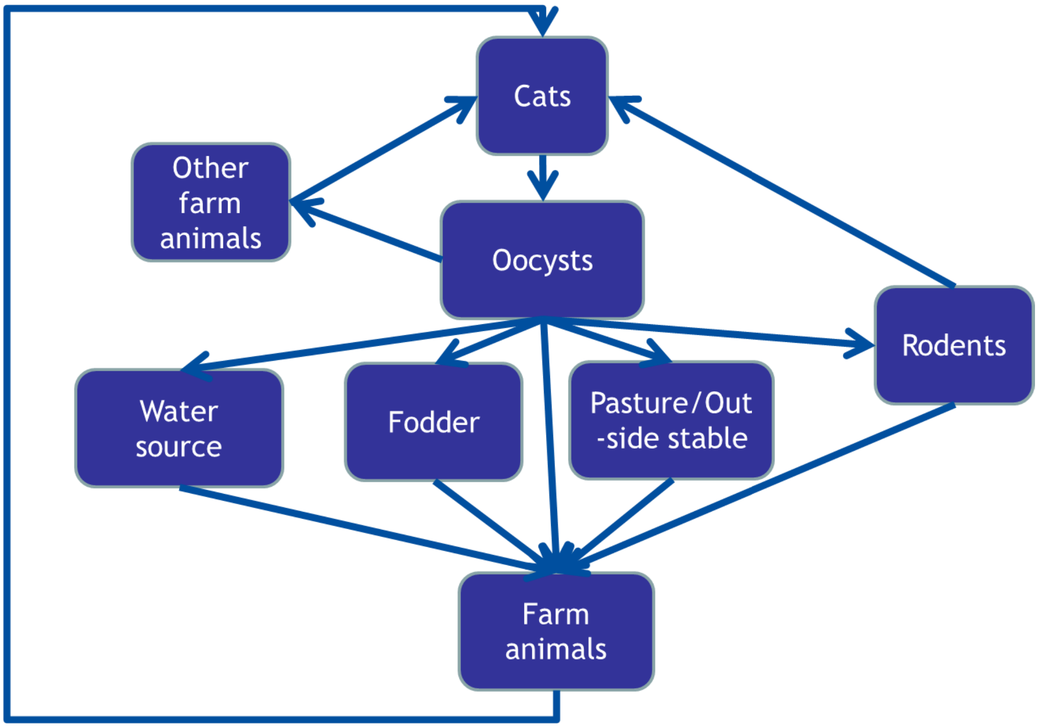

Figure 3: Putative routes of on-farm transmission of *T. gondii*

Almost all definitive host related variables were associated with an increased risk of *T. gondii* positivity of farm animals (pigs, small ruminants, cattle). These finding are in accordance with the biology of *T. gondii* and underline that the presence of cats plays a central role for *T. gondii* infection. The odds of positivity ranged between 1.37 – 11.3 once cats were present on farm (Appendix E, Supplementary Table S6). Not all cats might pose the same risk to animals and in a study in sheep only the presence of young cats was associated with risk (Refid 945). This might serve as an indication that young cats play a more important role for the cyclic transmission than older cats.

In two studies in pigs it was shown that the odds of positivity increased with the number of cats present on farm. Odds for positivity were higher for farms with more than two (Refid 1380) or more than three (Refid 492) cats (Appendix E, Supplementary Table S7). This dose-effect suggests that above mentioned relation between the presence of cats and *T. gondii* infection in pigs is very close. As mentioned above it has been shown that the direct observation of *T. gondii* oocysts in cat faeces, pig feed or soil was associated with an increased risk of infection in pigs indeed (Refid 1008) and it is very plausible that these oocysts could either directly or indirectly contribute to infections of farm animals. Oocysts shed by cats could contaminate food or water provided to the animals but could also contribute to infections of other intermediate hosts. Other intermediate host could serve as reservoirs for the definitive host but also contribute to the infection of farm animals as in the case of omnivorous livestock species like pigs. Cats with access to enclosures outside of stables, pastures or farmland could also increase the risk of infection via a contamination of these areas with oocysts.

Variables characterizing the level of confinement of animals and management intensity were analysed in pigs, small ruminants, cattle and chicken. Out of those variables which we regarded as indicators of a low level of confinement (Appendix E, Supplementary Table S8) all except one (2/2 in chicken; 13/14 in pigs) were associated with risk. It is likely that a high level of confinement provides protection and a low level of confinement is associated with risk. There was a large variation in the strength of association of a low level of confinement and positivity in these studies with Odds ratios or Relative risks ranging between 2.22 and 38.9.

However, in one study on pigs (Refid 1011) and one study on small ruminants (Refid 745) and in all studies from cattle (Note: these studies were included in a single reference [Refid 764]) a low level of confinement was associated with protection. These studies may indicate that “low level of confinement” is not always a risk factor especially in ruminants and that under certain circumstances a “low level of confinement” may confer protection. A potential reason could be that stables in which small-ruminants and cattle are reared are usually less confined than stables for pigs and chicken. Definitive and intermediate hosts of *T. gondii* may have easily access to these building in which cattle and small-ruminants are reared and higher population densities of definitive and intermediate hosts may occur especially in these buildings or at the places the fodder is stored. Under certain conditions this may cause higher levels of contamination in stables than on pastures or outside pens were cats und intermediate hosts of *T. gondii* may be more dispersed and prevail in lower densities. In one Norwegian study on sheep, “atypical grazing” was associated with risk and “atypical grazing” was defined as grazing of animals close to farm buildings (Refid 945).

The results of studies analysing the level of management intensity were often not in agreement with each other (all studies had been conducted in small ruminants.). A lower level of management intensity was not in all studies associated with an increased risk for *T. gondii* positivity (Appendix E, Supplementary Table S8). It can be assumed that “management intensity” is a variable which is not specific enough for characterizing the potential risks of a *T. gondii* transmission on farm. With this in mind it may not be surprising that in small ruminants a high level of management intensity (which may include in many cases also a higher level of confinement) was associated with an increased risk of *T. gondii* positivity (Appendix E, Supplementary Table S9).

Variables characterizing the likelihood of fodder contamination. Oocysts shed by cats may contaminate the fodder of animals. In addition, the presence of infected intermediate hosts or vectors in fodder may pose an infection risk, especially to omnivorous farm animals, e.g. pigs. In summary, most studies analysing factors which may serve as indicators for a possible fodder contamination revealed that these factors were associated with a risk for *T. gondii* positivity (Appendix E, Supplementary Table S10). In addition, those variables, indicating that a fodder contamination with *T. gondii* is unlikely, were associated with a reduced risk for farm animals being *T. gondii* positive in most studies (Appendix E, Supplementary Table S11).

Variables characterizing the likelihood of water contamination. Several severe outbreaks of human toxoplasmosis have been reported which clearly could be associated with contaminations of surface water with *T. gondii* oocysts. Consequently, providing surface water to farm animals is regarded as a risk factor and it is assumed that tap water should provide protection. However, when we summarized a number of risk factor studies there was no clear evidence that in general the use of tap water has a protective effect while the use of surface water is associated with risk (Appendix E, Supplementary Table S12). In both pigs and small ruminants a risk-effect for the use of surface water was observed. However, in small ruminants there was a number of studies that observed also a risk effect once tap water was used as a source of water for farm animals. Thus it has to be assumed that, at least for small ruminants, the availability of tap water was a confounder and that this variable was associated with other variables having contributed to the increased risk of the animals. It is possible that small ruminants provided with tap water are often located in or close to farm buildings populated by cats and other intermediate reservoir host species. In contrast those small ruminants with no access to tap water might be reared in remote areas with lower densities of cats and other intermediate hosts.

Rodent related variables. Rodents are regarded as important reservoir hosts for *T. gondii* and infected rodents harbouring tissue cysts may serve as direct sources of infection for omnivorous farm animals. The results of a number of studies in pigs are in accordance with this view and demonstrated that “No rodenticides used”, “No rodent control” and a “*T. gondii*-seroprevalence in house mice” was associated with risk in pigs (Appendix E, Supplementary Table S13). In this line are also studies reporting that “Rodent control” has a protective effect and an experimental study suggests that the duration of rodent control is associated with decreasing numbers of infected pigs. However, surprisingly there are also studies in pigs which revealed that “Rodent control” and “No presence of rodents” was associated with a risk. On one hand there was one study in small ruminants showing that the presence of rodents was associated with risk but on the other hand one study in small ruminants revealed that “Use of mouse poison” provided risk. A further study in pigs suggested that the “Use of cats or rodent proof containers” was superior to “Use of chemicals, traps or destruction of habitats against rodents”. This may serve as an indication that not every measure to control rodents is efficient and may in part explain the discrepancies between different studies.

Concluding remarks: In pigs and small ruminants a number of studies provide information on various potential risk and protective factors for *T. gondii* infections in farm animals. However, further studies are necessary to solve conflicting findings and to complete knowledge. There is a need for experimental studies to confirm the validity of findings of cross-sectional studies.

In other animal species including cattle, equids and poultry there were almost no studies available providing data on potential risk or protective factors for *T. gondii* infection. For these species it is essential that future cross-sectional studies provide a basis for further research on the epidemiology of *T. gondii* infection in these animal species.

CONCLUSIONS

The objective of this 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 four different topics. For each of these topics, conclusions are formulated at the end of the sections. In brief:

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

- Limited data were available for turkeys and horses.
- Predilection sites varied by species, but brain and heart ranked in the top 5 in pigs, sheep, goats, chickens, turkeys and horses.
- Predilection sites identified in cattle are different from those identified in the other species and the scores of the highest ranking tissues were low compared to the top scores in the other species.
- The results were used to select tissues for the experimental studies in cattle, pigs, horses and chickens. In pigs, horses and chickens the heart was selected as predilection site; in cattle the liver was selected. In cattle, pigs and horses the diaphragm was selected as representative of edible tissue; in chickens drumstick and lower leg muscle were selected.

2: the performance of available methods for detecting the presence and infectivity of *T. gondii* cysts, including their sensitivity and specificity:

- Mouse bioassay and PCR are the most commonly used methods for direct detection of *T. gondii* in livestock.
- Evaluation of the performance of direct detection methods using DNA dilution series, indicates that most PCR-based methods are able to detect the equivalent of one parasite. However, these results provide little information about the performance of PCR-based detection on tissue samples of animals harboring *T. gondii* tissue cysts as sampling and DNA isolation method will influence overall performance.
- The number of studies in which samples are spiked with tachyzoites, bradyzoites or tissue cysts prior to DNA isolation is limited, and studies that directly compare different types of detection methods (e.g. PCR in comparison to mouse or cat bioassay) using samples spiked with tissue cysts (with a quantified amount of bradyzoites, e.g. by qPCR) do not exist.
- From studies that present matched results with two or more direct detection methods for experimentally or naturally infected animals, it is clear that cat bioassay performs best, followed by mouse bioassay. PCR can perform similarly to mouse bioassay depending on sampling and protocol details. Detection based on microscopy lacks sensitivity.

3: the relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues:

- There is a lack of information especially for turkeys, horses, cattle and goats.
- Current data suggest concordance between detection of antibodies to *T. gondii* and direct detection of the parasite in pigs, small ruminants and chickens. Direct detection was positive for 34.9% (goats) to 58.8% (pigs) of seropositive animals. Absence of antibodies does not guarantee that

meat is free of *T. gondii*; direct detection was positive in up to 4.9% (pigs) of seronegative animals.

- The data available on cattle and horses suggest a lack of concordance, with a low recovery rate in seropositives and similar rates of direct detection of the parasite in seronegatives and seropositives.

4: the relationship between the on-farm risk factors and *T. gondii* infection in pigs, cattle, small ruminants, poultry and equids:

- In pigs and small ruminants a number of studies provide information on various potential risk and protective factors for *T. gondii* infections in farm animals. In other animal species including cattle, equids and poultry there were almost no studies available.
- The following conclusions were made for factors considered biologically relevant:
  - Variables related to the presence of cats or on farm detection of *T. gondii* oocysts were always identified as risk factors (pigs, small ruminants).
  - Most variables characterizing the likelihood of fodder contamination suggest an increased risk when likely and protection when unlikely (pigs, goats).
  - Low level of confinement was in most studies associated with increased risk (pigs, chickens), but also with protection in a few studies (pigs, cattle).
  - Variables suggesting a likely transmission via rodents were associated with risk (pigs, sheep). However, when variables suggested unlikely transmission via rodents, this revealed either risk or protection (pigs, sheep).
  - Variables characterizing the possibility of water contamination or the level of management intensity revealed no clearly directed effect regarding the risk of farm animals being *T. gondii* positive.
- Further studies are necessary to solve conflicting findings and to complete knowledge. There is a need for experimental studies to confirm the validity of findings of cross-sectional studies.

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APPENDICES

Appendix A. WP2 *a priori* protocol

Systematic review on relationship between presence of *T. gondii* and infectivity, detection tests, anatomical distribution of the main livestock species

Contents

1 Background

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, 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., 2011), 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.

The *a priori* protocol described was designed to perform the following specific tasks (as described by EFSA):

To carry out an extensive literature search and review of available data on *Toxoplasma gondii* in meat of the main livestock species (e.g. pigs, ruminants, poultry, and solipeds) to provide information on:

Task 1: the relationship between seroprevalence in the main livestock species and presence and infectivity of *T. gondii* cysts in their meat and other edible tissues;

Task 2: the performance of available methods for detecting the presence and infectivity of *T. gondii* cysts, including their sensitivity and specificity; and

Task 3: the anatomical distribution of the cysts in meat and other edible tissues, to inform the optimal sampling choice(s) for slaughtered animals for optimisation of detection.

To facilitate the review process the following terms are defined more precisely:
Seroprevalence: percentage of animals positive for **antibodies** against *Toxoplasma gondii* determined in **serum or meat juice**.
Presence and infectivity of T. gondii cysts: specific detection of *T. gondii* with any direct detection method is accepted (e.g. cat or mouse bioassay, in vitro cultivation, PCR or antigen-ELISA or other method for antigen detection, in case of microscopy the method for differentiation from other cyst-forming protozoa needs to be described). These methods are valued differently for their ability to discriminate infective and non-infective parasites, as well as for their ability to differentiate tissue cysts from other parasite-stages.
Meat and other edible tissue: all tissues will be considered
Pigs: domestic pigs (*Sus scrofa domesticus*, not farmed wild boar)
Bovines: domestic cattle (only *Bos taurus* breeds, not buffalo)
Small ruminants: domestic sheep (*Ovis aries*) and domestic goats (*Capra aegagrus hircus*)
Poultry: limited to chicken (*Gallus gallus domesticus*) and domestic turkey (*Meleagris gallopov*a)
Horses: *Equus ferus caballus* (also include ponies)

Europe (in relation to European husbandry): EU member states, territories belonging to EU countries but outside the continent are excluded.

2 Systematic Review approach

The systematic review will follow a predefined protocol based on Cochrane guidelines www.cochrane.org and EFSA guidance www.efsa.europa.eu . The protocol includes four main steps: identification, screening, data extraction andquality assessment.

2.1 Review objectives

To identify, appraise and summarize available scientific evidence on:

- The relationship between detection of antibodies and presence of infectious *T. gondii* tissue cysts in meat and other edible tissues of pigs, bovines, small ruminants, poultry and horses (task 1).
- The performance (sensitivity and specificity) of available methods for detecting the presence and infectivity of *T. gondii* tissue cysts in meat and other edible tissues of pigs, bovines, small ruminants, poultry and horses (task 2).
- The anatomical distribution of *T. gondii* tissue cysts in meat and other edible tissues of pigs, bovines, small ruminants, poultry and horses (task 3).

These three tasks are combined in one WP, as all three rely on studies reporting results with a direct detection method. Therefore, the same search strategy can be applied to retrieve records. Eligibility for the three tasks will then be scored separately, as studies for task 1 require the use of a direct and an indirect detection method; studies for task 2 require evaluation of the direct detection method, for example by comparison with a gold standard status based on indirect or direct detection or experimental infection; and studies for task 3 require that the tissue tested is defined.

2.2 Identification of relevant published papers

2.2.1 Information sources

Bibliographic searches will be carried out using the following databases:

- MEDLINE
- EMBASE
- BIOSIS

The Systematic Review will be carried using Distiller SR provided by EFSA.

PhD thesis searches will be carried out using the following databases:

- www.worldcat.org
- www.ubka.uni-karlsruhe.de/kvk_en.html.

Grey literature will not specifically be searched for. However, any of the working group members is aware of additional studies that should be included, those studies will be added to the list of retrieved records. These studies can be in any language as long as two reviewers will be able to perform the screening.

2.2.2 Search strategy

The search concept covers the following review questions:

- What is the relationship between seroprevalence and presence and infectivity of *T. gondii* cysts in meat and other edible tissues? (Q1)
- What is the performance of available methods for detecting the presence and infectivity of *T. gondii* cysts in meat and other edible tissues? (Q2)
- What is the anatomical distribution of the cysts in meat and other edible tissues? (Q3)

For the initial identification of relevant studies we consider development (till mid-January) of specific search terms on the following key subjects:

- **Toxoplasma** as main topic/ pathogen of interest,

AND

- **animals** (pigs, bovines, small ruminants, poultry and horses) as target population

AND

- **detection** (method to detect infection or presence of cysts)

OR

- **presence (**antibody or *T. gondii* cysts)

The following technical items will also be taken into account:

- UK and US spelling and terminology,
- Synonyms - e.g. cattle, cow, bovine, ruminants etc.
- thesaurus for subject searching (Medical Subject Headings system -'MeSH') articles indexed through controlled vocabulary
- Boolean operators (AND, OR, NOT),
- truncation (*) – e.g. Toxoplasm*
- and wild cards (#) – e.g. Toxopl#m*
- language restricted to English, German and French
- there will be no limitation on publication date

Databases will be searched using keywords associated with the Boolean operators **AND/OR.** The asterisk (*), when used, expanded the search by looking for words with similar prefixes (i.e. toxoplasm* will search for Toxoplasma, toxoplasmosis). Different combinations will be tailored for each electronic database in order to narrow the amount of results retrieved but at the same time maximizing the number of relevant studies. The search strategy for Medline is presented in Appendix A.

Retrieved records will be imported in EndNote, and checked for duplicates which will be removed. Next, records will be imported into Distiller SR, a specific program for reference managing and evaluation (Distiller SR) will be used. A second check for duplicates will be performed using Distiller SR. When outcomes overlap, all duplicates articles will be removed.

2.3 Study selection

Initially, the selection protocol will be validated for reliability and reproducibility, using a subset of publications already identified as either relevant or not relevant to the objective. Next, studies identified using the search strategy for bibliographic databases as well as those identified through thesis databases and identified grey literature will be assessed against the inclusion and exclusion criteria for relevance and eligibility. The screening will be performed in two stages. First, titles and abstracts will be screened for relevance. Next, full-text reports of records found relevant will be screened for eligibility.

2.3.1 Screening of titles and abstracts for relevance to the review question

All unique records will be divided over the WP-members (2 reviewers per record), and after a title screen (Is this record potentially relevant?) the relevance for screening of full text will be determined based on title and abstract. If the first reviewer considers a record relevant, it will be included in the full-text screening. When the reviewer does not consider the record relevant, the record will be screened by the second reviewer. If the second reviewer considers the record relevant it will be included in full-text screening, if not, the record will be added to a list of non-relevant records. If no abstract is available or the abstract is too vague, the full text version will be retrieved and screened. The titles and abstracts will be screened for relevance using the following criteria:

Inclusion criteria

- Peer reviewed scientific publications published or in press, or PhD/doctoral thesis
- Reports of original data as a primary source (e.g. remove reviews, editorials or letters to the editors without the original data)
- Paper addresses key elements in the review questions
    - Studies concerning the pathogen of interest (*T. gondii*, all isolates), no restrictions on infection route (natural and experimental infection using tachyzoites, tissue cysts, bradyzoites, oocysts or sporozoites).
    - At least one of the animal species of interest is included.
        - o Host species: restricted to food animals most commonly consumed in Europe: pigs (domestic only), bovines (*Bos taurus* breeds), small ruminants (domestic sheep and goats), poultry (domestic chickens and turkeys) or horse and ponies.
    - At least one tissue (no restrictions on type of tissue) was tested using a direct detection method
        - o Direct detection method: any direct detection method is accepted (e.g. cat or mouse bioassay, in vitro cultivation, PCR or antigen-ELISA or other method for antigen detection) , publications that report results with only indirect detection methods are not (these may still be suitable for WP3)

2.3.2 Examining full-text reports for the eligibility of studies

Any of the inclusion criteria (2.3.1) that could not be properly evaluated based on title and abstract alone will now be evaluated based on the full-text. Additional exclusion criteria that are evaluated in this phase are:

- Full-text could not be obtained within two weeks after selection for full-text screening was completed for all records.
- Publication contains only duplicated data.

Studies that fail to meet inclusion criteria or meet exclusion criteria at this point will be excluded. Screening will be performed independently by two members, and, in this phase, doubts or disagreements will be resolved by discussion with the WP-leader and documented. A flow-chart with studies included/excluded at each step of the screening process, a list of studies not found eligible (with reasons), and a list of studies not available in full-text will be provided. Where several records refer to the same study these will be grouped together and screened together as one study unit.

2.3.3 Task-specific inclusion criteria

At the end of the full-text screening, but before starting data collection a task identification form will be filled in by the reviewers. Based on the availability of the following information, the applicable task(s) are identified and a specific set of forms will become available during the data collection phase:

Question 1 (correlation):

- Both a direct and indirect detection method are used

Question 2 (test characteristics):

- The performance of a direct detection method is evaluated (e.g. by testing spiked samples, samples from experimentally infected animals, or by comparison to another method).

Question 3 (anatomical distribution):

- Tissue tested by direct detection method are defined

3 Data collection and entry into evidence tables

Data will be extracted from all papers considered as eligible. The information will be collected in standardised electronic forms in DistillerSR and subsequently imported into spreadsheets (Access, XLS or other compatible with EFSA requirements).

Information about test characteristics that affect external applicability (e.g. sampled population) and internal validity (e.g. diagnostic accuracy of the test used) will be collected in addition to information about the results. For each eligible study data will be collected and entered by one member of the work package and verified by another member. Discrepancies will be resolved by discussion including the WP leader. The same member will be responsible for the evaluating and assessing the relationship between presence of *T. gondii* and infectivity, the tests available for detecting the presence and infectivity of tissue cysts, and the anatomical distribution. Based on the task identification, only applicable forms will show up in distillerSR.

Tables will be prepared including detailed information listed in Appendix B.

4 Assessment of methodological quality

All papers that pass the relevance screening will be subject to assessment of their quality.
Aspects of the design, execution, analysis and reporting of a study that may lead it to give a biased result will be evaluated by two reviewers using a pre-defined checklist in distillerSR. . A statistician and epidemiologists in consortium will be consulted for set-up of the checklist, and the checklist will be discussed in the whole WP. The checklist for methodological quality will focus on internal and external validity. Internal validity is reached when the study results reflect reality among the animals under study, whereas external validity is reached when the study results are reasonably generalised to the broader reference population. The main biases affecting validity are confounding, selection bias and information bias. Our checklist will contain criteria that provide information on the following aspects:

- Sampling strategy (sample size, selection process, randomization)
- Comparability of the sampled animal population to European food animals (age, husbandry conditions)
- Comparability of experimental infection to natural infection (infection route, parasite-stage, dose, time post infection).

- Validity of the diagnostic method, including the analytic and diagnostic sensitivity and specificity
- Feasibility of using the detection method to discriminate the different parasite-stages and infective and non-infective parasites
- Completeness of data

Part of the information needed to assess these issues is already collected on other data collection forms, the remainder will be collected on a separate form. The information on the criteria of interest is extracted from distillerSR and collected in tables per quality-related issue. Data will be analysed in the subgroups and exchanged for peer-review between the subgroups. A statistician and epidemiologists in consortium will be will be available for advice on the assessment.

5 Presenting data and results

A flow diagram for identified records will be presented according to the PRISMA statement (Fig. 1). However, we do not plan to perform a meta-analysis. The data will be presented in tables and/or charts, and interpreted and discussed narratively. The presentation will include the characteristics of the included primary studies, the data collected from the primary studies, and the results of analyses carried out on those data (e.g. assessment of methodological quality).

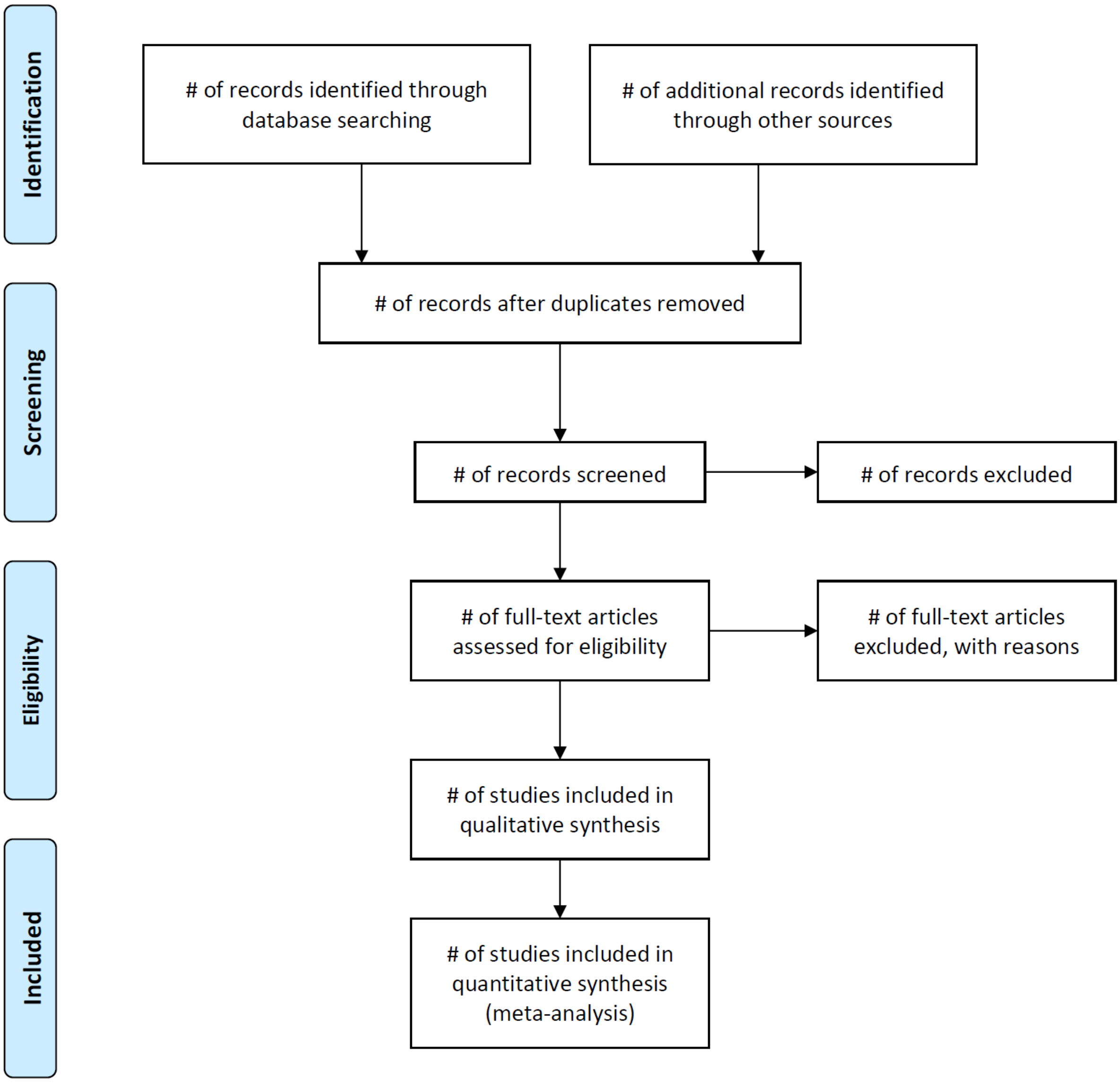

Fig. 1 PRISMA flow diagram for reporting the number of records identified, included and excluded, and the reasons for exclusions

6 Interpreting results and drawing conclusions

Epidemiologists in the consortium will, in consultation with a statistician, decide on the weight of data in the interpretation. The following issues will be addressed in the discussion and conclusions sections of the systematic review:

1. The quantity of evidence (e.g. number of papers and number of subjects).
2. The quality of the evidence. This will involve considerations of study methodological quality, heterogeneity, precision of parameter or effect estimates, and potential biases.

3. Interpretation of the results. The statistical and the biological significance of the findings will be interpreted with a clear explanation of all assumptions made. In cases where very few relevant data are found, knowledge gaps will be characterised and reported.
4. Any potential limitations of the review process.
5. Agreements or disagreements with other studies or reviews.

7. References

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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.

Appendix A: Search strategy to retrieve records from Medline

1 exp Sus scrofa/ (12743)

2 exp Sheep/ (105558)

3 exp Goats/ (25576)

4 exp Cattle/ (298530)

5 exp Chickens/ (99553)

6 exp Turkeys/ (9066)

7 exp Horses/ (57408)

8 exp Food/ (1070672)

9 exp *Birds/ (83464)

10 exp Antibodies/ (720132)

11 exp Immunoassay/ (453924)

12 exp Immunologic Tests/ (415648)

13 exp Immunoprecipitation/ (95585)

14 exp Polymerase Chain Reaction/ (396500)

15 exp Biological Assay/ (34185)

16 (bioassay$ or (bio adj assay$)).ti,ab. (31748)

17 exp Toxoplasma/gd, im, ip, ps, py [Growth & Development, Immunology, Isolation & Purification, Parasitology, Pathogenicity] (8013)

18 exp Toxoplasmosis/ (17241)

19 exp Swine Diseases/ (24913)

20 exp Cattle Diseases/ (60247)

21 exp Sheep Diseases/ (24003)

22 exp Bird Diseases/ (37196)

23 exp Horse Diseases/ (23060)

24 exp Goat Diseases/ (3901)

25 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 (1579518)

26 17 or 18 (19259)

27 10 or 11 or 12 or 13 or 14 or 15 or 16 (1691927)

28 19 or 20 or 21 or 22 or 23 or 24 (162220)

29 (25 or 28) and 26 and 27 (1045)

30 limit 29 to "review articles" (26)

31 29 not 30 (1019)

32 limit 31 to (english or french or german) (965)

Appendix B: Data planned to be extracted from records

Part 1: Sampling information							
RefID							
First author's family name							
Publication year							
Research period							
Research location (Continent, country, Region/state/province)							
Species:	*Pig*	*Cattle*	*Sheep*	*Goat*	*Chicken*	*Turkey*	*Horse*
Animal category	Age (if not defined, choose category):						
	Suckling piglets (up to 5 weeks)	Calf	Lamb	Kid	Broiler	Broiler	Foal
	Weaned piglets (6 to 12 weeks)	Heifer	Breeding ewe, not lambed	Dairy goat, not lambed	Breeding animal	Laying hen	Adult
	Fattening pigs (13-35 weeks)	Dairy cow	Breeding ewe, lambed	Dairy goat, lambed	No information	Breeding animal	No information
	Gilts	Beef cattle	Breeding ram	Dairy goat, not specified		No information	
	Sows	No information	Breeding animal, not specified	Breeding stock			
	Boars		No information	No information			
	No information						

Infection method (natural or experimental)					
Characteristics of experimental infection (for each group)	Infection route: oral/intramuscular/subcutaneous/intravenous/other:/not specified				
	Parasite stage: tachyzoite/bradyzoites/oocysts/excysted sporozoites/other:				
	Dose:				
	Strain of T. gondii:				
	Time between infection and euthanasia:				
	Other characteristics that define the experimental groups:				
Characteristics of natural infection	Strains of T. gondii (if determined):				

Part 2: Direct detection method: general information, performance and anatomical distribution (all records eligible for WP2)							
Which method(s) were applied	*Bioassay*	*PCR*	*in vitro isolation/ tissue culture*	*immunohistochemistry/immunofluorescence*	*microscopy without staining/ aspecific staining*	*antigen-ELISA*	*other:*
Specifics of the method	mouse/cat	PCR target	cell line	describe any characteristics that may influence the detection limit of this method			
	artificial digestion y/n, and select options for yes (pepsin/trypsin)	single round conventional PCR/ (semi)nested conventional PCR/ qPCR	homogenisation or digestion (y/n and select options)				

	#mice or cats/sample	DNA isolation method (select from options)	total fraction of digest that was inoculated on cells				
	method of detection in mouse/cat (select from options)	Controls used in PCR (select from options)	days of cultivation				
			method to detect amplification				
Detection limit (if reported)							
Information on cross-reactivity							
Sensitivity (if reported)							
Specificity (if reported)							
<u>Fill in (new column per combination of method, tissue and exposure group):</u>							
Method							
Tissue							
Starting volume of the sample (/tissue in g or not specified)							
Exposure group (exp infected with details/negative control/seropositive/seronegative/clinical case/general population)							
No of animals							
No of positive animals (at least one of the tissue samples)							
Quantitative information on positives (positive samples/tissue, positive mice/tissue or parasite contrations/numbers)							

Part 3: Indirect detection method (only if record reports results with an indirect detection method)								
Which method(s) were applied	*ELISA*	*MAT*	*Toxoscreen-DA*	*IFAT*	*IHAT*	*Latex agglutination*	*Western blot*	*Other:*
Commercial (provide name) or in-house								
Type of sample (serum/meat juice/plasma/other:)								
What sample dilution(s) were tested								
Type of antigen (fixed intact parasites/parasite lysate: sonication, freeze-thaw, detergent, or other/native pruified protein, define:/recombinant protein, define:)								
What cut-off value was used								
What was the cut-off value based on (manufacturer/reference to literature/mean for negative controls plus standard deviation(s)/ROC curve/binary mixture model/direct comparison to results with other assay(s)/bayesian (latent-class) analysis/other:/not specified)								
Sensitivity (if reported)								
Specificity (if reported)								
<u>Fill in (new column per combination of method, sample type and exposure group):</u>								
Method								
Sample type								
Exposure group (exp infected with details/negative control/animal category/clinical case/general population)								
No of animals								
No of positive animals								
Reported seroprevalence with CI, SE or SD								

Part 4: Results relationship (only for records that report results of with direct and indirect detection method)					
Are direct and indirect detection results that are matched per animal presented? Y/N					
If matched results are presented, is a correlation measure presented (no/yes: kappa-value, sensitivity and specificity,other:) and, if yes, report value:)					
If raw data for the matched results are presented, fill in cross-tabulation (animal-level):					
		I1, pos	I1, neg	I2, pos	I2, neg
	D1, pos				
	D1, neg				
	D2, pos				
	D2, neg				

Appendix B. WP3 *a priori* protocol

Systematic review on the relationship between the on-farm risk factors and *Toxoplasma gondii* infection in pigs, bovines, small ruminants, poultry and horses

Contents

1. Background

T. gondii is an important zoonotic protozoon, infecting a large variety of warm-blooded vertebrates [1]. In infected humans, *T. gondii* can be vertically transmitted, which may cause death or clinical illness in the foetus, the new born child or later on in prenatally infected persons, even if they seem to be healthy at birth. Immunocompromised humans may suffer from toxoplasmosis after a postnatally acquired or re-activated latent *T. gondii* infection [2].

The main cause of postnatal infection in humans seems to be ingesting raw or not sufficiently treated meat containing tissue cysts [3]. The oral uptake of sporulated oocysts is regarded as another important route of postnatal infection [3]. *T. gondii* oocysts are transiently shed by the definitive hosts of the parasite, i.e. felids, in particular domestic cats, upon oral infection [4] [5].

In Europe predominantly pigs, poultry, cattle and small ruminants are used for meat production. These animal species used for meat production in Europe are potential intermediate hosts for *T. gondii*, although their relative importance as source of infection for humans is regarded variable. For example, the relative importance of pork is considered high while that of cattle has been assumed to be low in the past [6].

To reduce the risk of humans to become infected with *T. gondii* either congenitally or post-natally it is essential 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 [7] [8].

The objective of the systematic literature review is to identify, appraise and summarize available scientific evidence addressing the following Review Question (RQ):

What is the relationship between the on-farm risk factors and *T. gondii* infection in pigs, bovines, small ruminants, poultry and horses?

2. Systematic Review Approach

The systematic review follows a predefined protocol based on Cochrane guidelines www.cochrane.org and EFSA guidance www.efsa.europa.eu. The protocol contains the following main steps: identification, screening, quality appraising for eligibility and data extraction.

2.1. Review objective

The objective of the systematic literature review is to identify, appraise and summarize available scientific evidence addressing the following Review Question (RQ):

What is the relationship between the on-farm hypothetical risk factors and *T. gondii* infection in pigs, bovines, small ruminants, poultry and horses?

2.2. Identification of relevant published papers

2.2.1. Information sources

Bibliographic searches will be carried out using the following databases:

- MEDLINE
- EMBASE
- BIOSIS

PhD/doctoral thesis searches will be carried out using the following databases:

- www.worldcat.org
- www.ubka.uni-karlsruhe.de/kvk_en.html.

Apart from checking the references of identified (review) papers other grey literature than PhD/doctoral theses will not specifically be searched for. However, any of the working group members is aware of additional studies that should be included, those studies will be added to the list of retrieved records. These studies can be in any language as long as two independent reviewers will be able to perform the screening.

2.2.2. Search strategy and identification

The search concept covers the review question (What is the relationship between the on-farm risk factors and *T. gondii* infection in pigs, bovines, small ruminants, poultry and horses?).

For the initial identification of relevant studies we consider the development of specific search terms on the following key subjects:

- **Toxoplasma** as main topic/ pathogen of interest,

AND

- **animals** (pigs, bovines, small ruminants, poultry and horses) as target population

AND

- on farm risk factors

The following technical items will also be taken into account:

- UK and US spelling and terminology,
- Synonyms - e.g. cattle, cow, bovine, ruminants etc.
- thesaurus for subject searching (Medical Subject Headings system -'MeSH') articles indexed tough controlled vocabulary
- Boolean operators (e.g. AND, OR, NOT),
- truncation (*) – e.g. Toxoplasm*
- and wild cards (#) – e.g. Toxopl#m*
- language restricted to English, German and French

Databases will be searched – if appropriate – using keywords associated with the Boolean operators **AND/OR.** The asterisk (*), when used, expanded the search by looking for words with similar prefixes (i.e. toxoplasm* will search for Toxoplasma, toxoplasmosis). Different combinations will be tailored for each electronic database in order to narrow the amount of results retrieved but at the same time maximizing the number of relevant studies.

Search period will be limited to publications in last 20 years (i.e. publications from 1994 onwards) to address most recent knowledge in the topic.

Retrieved records will be imported in EndNote and duplicates will be removed. Next, records will be imported into Distiller SR (if made available by EFSA), a specific program for reference managing and evaluation (Distiller SR). A second check for duplicates will be performed using Distiller SR and duplicates removed.

2.3. Study selection

Initially, the selection protocol will be validated for reliability and reproducibility, using a subset of publications already identified as either relevant or not relevant to the objective. Next, studies identified using the search strategy for bibliographic databases as well as those identified through thesis databases and identified grey literature will be assessed against the inclusion and exclusion criteria for relevance and eligibility. The screening will be performed in two stages. First, titles and abstracts will be screened for relevance. Next, full-text reports of records found relevant will be screened for eligibility.

2.3.1. Screening of titles and abstracts for relevance to the review question

All unique records will be divided over the WP-members (2 reviewers per record), and based on title and abstract the relevance for screening of full text will be determined. If the first reviewer considers a record relevant, it will be included in the full-text screening. When the reviewer does not consider the record relevant, the record will be screened by the second reviewer. If the second reviewer considers the record relevant it will be included in full-text screening, if not, the record will be added to a list of non-relevant records. If no abstract is available or the abstract is too vague, the full text version will be retrieved and screened. The titles and abstracts will be screened for relevance using the following criteria:

Inclusion criteria:

- Peer reviewed scientific publications published or in press
- Reports of original data as a primary source (e.g. remove reviews, editorials or letters to the editors without the original data)
- Paper addresses the key elements in the review question:
  - Studies concerning the pathogen of interest (*T. gondii*, all isolates),
  - Only natural infection routes
  - At least one of the animal species of interest is included.

Host species: restricted to food animals most commonly consumed in Europe: pigs (domestic only), bovines (*Bos taurus* breeds), small ruminants (domestic sheep and goats), poultry (domestic chickens and turkey) or horse.

Exclusion criteria:

- Descriptive studies
- Studies limited to experimental infection with *T. gondii*.
- Case reports,
- Studies missing data-driven assessments of potential on-farm risk and protective factors for *T. gondii* infection in farm animals.
- Duplicated data.
- Full-text could not be obtained within one month after selection for full-text screening was completed for all records.
- Risk factors studied are not applicable to European husbandry (e.g. related to incomparable climatic or geographical conditions)

During screening of title and abstract a note will be made of the species, to allow division over the species-specific WP-members in the full-text screening.

2.3.2. Examining full-text reports for the eligibility of studies

Any of the inclusion and exclusion criteria (2.3.1) that could not be properly evaluated based on title and abstract alone will now be evaluated based on the full-text. Studies that fail to meet inclusion criteria or meet exclusion criteria at this point will be excluded. For the remaining studies it is considered whether population, exposure, comparator, outcome and study design (PECOS) are reported (Yes/No).

The population being addressed (P):

Farm animals: pigs (*Sus scrofa domesticus*), bovines (*Bos taurus*), small ruminants (sheep, *Ovis aries*; goats, *Capra aegagrus hircus*), poultry (Chicken, *Gallus gallus domesticus*; turkey, *Meleagris gallopavo*) and horses (*Equus ferus caballus*). There will be no geographic restriction. However some studies may be excluded if data were retrieved in regions where climatic factors are not similar to Europe.

Exposure (E)

- evidence of risk factors to which the population has been exposed on farm

The comparators (C):

- consider a reference scenario against which the outcome or exposure can be compared
    - controls – animals without disease or as a reference group in the study; or
    - not exposure – animals with a lack of exposure to the factor of interest; or
    - reference situation – animal status at a point prior to exposure to risk factors
    - a cumulative effect (dose –relation) between level of risk factor and outcome

The main outcome or endpoint of interest (O):

- the reported strength of association or impact (effect) of particular risk factor to infection with *T. gondii*

To create an overview of potential risk and protective factors for animal infections with *T. gondii*

The study designs chosen (S):

- Observational epidemiologic studies
    - case-control,
    - cohort studies,
    - cross-sectional and
    - studies with hybrid design.
- Experimental studies (e.g. vaccination as field trial within environmental risk factors)
    - Field trials

Two independent reviewers will screen papers for completeness of reporting the PECOS characteristics. If both reviewers conclude that a study reports all data, the study will be considered relevant for the data collection phase. In case of disagreements or doubts, inclusion of the study will be discussed with the WP-leader or another member of the review team. A list of studies found non-relevant, in which reasons for exclusion are reported, will be compiled. Several members of the review team will independently assess a subset of the studies (~10%) classified as relevant and non-relevant. If results indicate that reviewers are inconsistent in their assessment (e.g. in > 40%), the discrepancies will be discussed and the criteria will be clarified or modified.

3. Data collection and entry into evidence tables

Data will be extracted from all papers considered as eligible. The information will be collected in standardised electronic forms in DistillerSR and subsequently imported into spreadsheets (Access, XLS or other compatible with EFSA requirements). All relevant information addressing the review questions (e.g. PECOS), the characteristics of the study (study design, sample size, period of follow up) and risk of bias will be recorded, in addition to information about the results, whenever applicable. For each eligible study data will be collected and entered by one of the species-specific member of the work package and verified by the other species-specific member (roles reversed in WP2). Discrepancies will be resolved by discussion including the WP leader.

Tables will be prepared including the following sections:

- Reference identification (unique identifier)
- Author's name (Family) and publication year
- Time (when was the study performed?)
- Location (where was the study population located?)
- Livestock animals (animals of interest, included in the review questions)
- Age category
- Number of animals
- Reference scenario (please refer to the comparators in 2.3.2.)
- Reported outcomes (see options in Table2)
- Study design
- Quality appraisal
    - correct study application (yes /no, + major issues)
    - correct reporting (yes/no +major issues)
    - potential bias (see Table 1)

4. Assessment of methodological quality

Quality appraising
All papers that pass the relevance screening will be subject to assessment of their quality. The full text of the articles will be evaluated by two reviewers using a pre-defined checklist.
In order to provide as consistent and unbiased quality appraisal as possible a check list with questions (Quality form) is developed in line of (adapted) STROBE, PRISMA, EFSA guidelines and others.

Check list Questions

- Is the method clear, transparent whereby results are reproducible?
- Are the elements of PICOS/ PECOS identifiable (easily)?
- What study design was utilized?
    - Observational: cross sectional
    - Observational: cohort
    - Observational: case-control
    - Observational: case-control with genetic evidence
    - Experimental
    - Other
- Are we confident that study design is applied correctly?
    - To consider in the observational studies
        - risk of selection bias (see Table 1)
        - risk of information bias
        - other misclassification
        - adequate control of confounding

    - any other relevant issues
  - To consider in the field experimental studies
    - allocation adequately
    - blinding
    - incomplete outcome data: loss to follow-up, analysis etc
    - selective reporting of results
    - any other relevant issues
- Does it present a sufficient quality of reporting?
  - Does it report sample size calculation?
  - Does it report loss to follow up in cohort studies?
  - What is the source of recruiting the controls?
  - Do the results allow checking of analysis?
  - Does it apply randomization?

Table 1. Risk of bias and interpretations

Risk of bias	Interpretation	Within a study
Low risk of bias	Bias, if present, is unlikely to alter the results seriously	Low risk of bias for all key domains
Unclear risk of bias	A risk of bias that raises some doubt about the results	Low or unclear risk of bias for all key domains
High risk of bias	Bias may alter the results seriously	High risk of bias for one or more key domains

adapted from Cochrane Handbook, 2011

5. Presenting data and results

A flow diagram for identified records will be presented according to the PRISMA statement (Fig. 1). However, we do not plan to perform a meta-analysis. The data will be presented in tables and/or charts, and interpreted and discussed narratively. The outcome measuring characteristics may differ (see

Table 2) in the studies and that variation would be taken into account in data extraction.

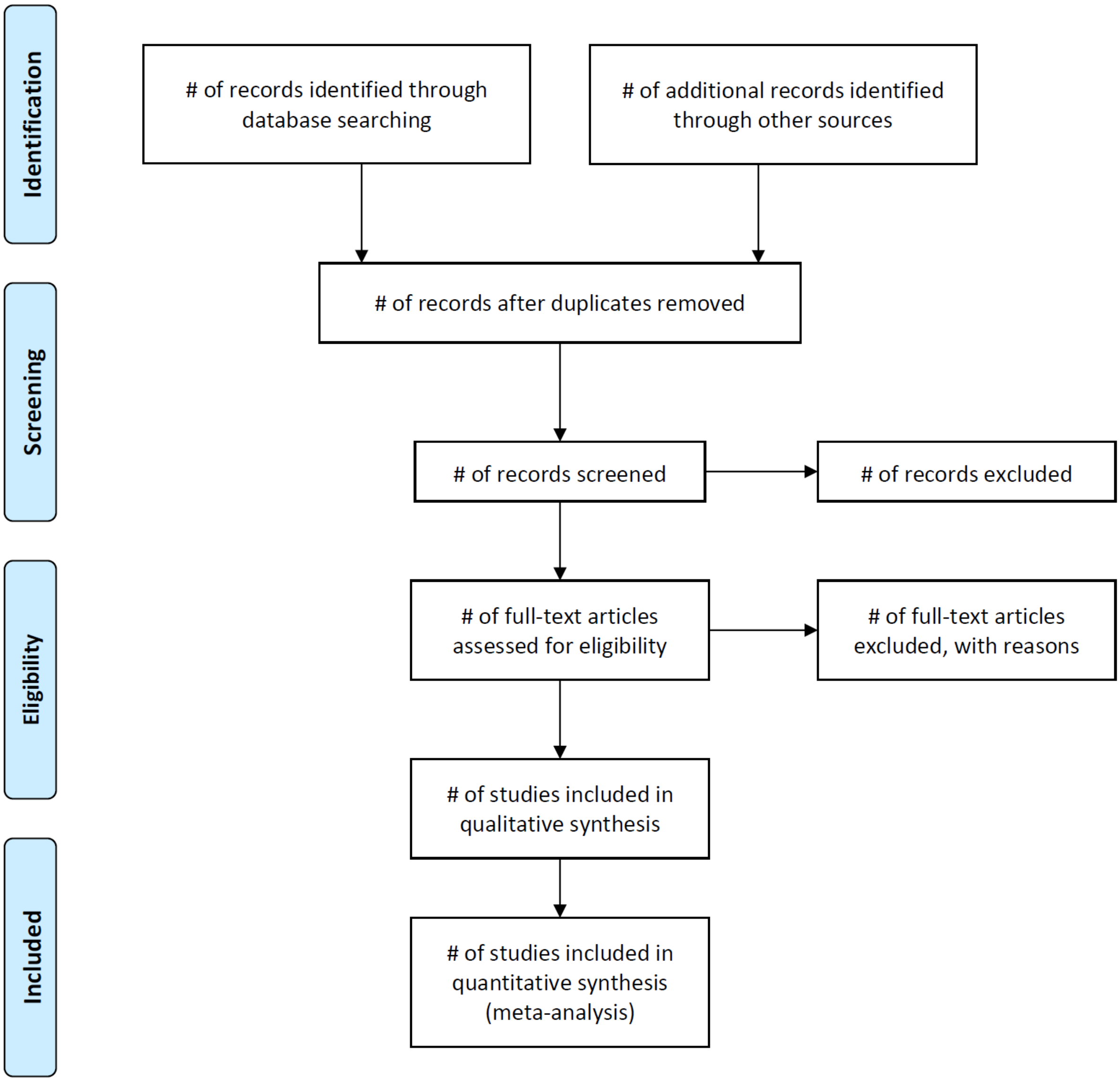

Fig. 2 PRISMA flow diagram for reporting the number of records identified, included and excluded, and the reasons for exclusions

Table 2. Example of outcome data

Data type	Dichotomous outcome	Continuous outcome
Summary estimate	Point estimate; RR, OR, RD, IR Estimate precision : SE or CI 95%	Point estimate: mean difference (MD) Estimate precision : SE or CI 95%
Group	Values for exposed and control groups	Number, mean and standard deviation in each group from which the MD and its SE can be computed
Individuals	Raw data – outcome Yes/No (0 or 1) and characteristics for each subject	Raw data – outcome (continuous scale) and characteristics for each subject

Adjusted from Dohoo et al 2007

6. Interpreting results and drawing conclusions

Epidemiologists in the consortium will, in consultation with a statistician, decide on the weight of data in the interpretation. The following issues will be addressed in the discussion and conclusions sections of the systematic review:
1. The quantity of evidence (e.g. number of papers and number of subjects).
2. The quality of the evidence. This will involve considerations of study methodological quality, heterogeneity, precision of parameter or effect estimates, and potential biases.
3. Interpretation of the results. The statistical and the biological significance of the findings will be interpreted with a clear explanation of all assumptions made. In cases where very few relevant data are found, knowledge gaps will be characterised and reported.
4. Any potential limitations of the review process.
5. Agreements or disagreements with other studies or reviews.

7. References

1. Dubey JP (2010) Toxoplasmosis of animals and humans. Boca Rotan: CRC Press.
2. Montoya JG, Liesenfeld O (2004) Toxoplasmosis. Lancet 363: 1965-1976.
3. Cook AJC, Gilbert RE, Buffolano W, Zufferey J, Petersen E, et al. (2000) Sources of toxoplasma infection in pregnant women: European multicentre case-control study. British Medical Journal 321: 142-147.
4. Davis SW, Dubey JP (1995) Mediation of immunity to Toxoplasma gondii oocyst shedding in cats. Journal of Parasitology 81: 882-886.
5. Dubey JP (1995) Duration of immunity to shedding of Toxoplasma gondii oocysts by cats. Journal of Parasitology 81: 410-415.
6. Kijlstra A, Jongert E (2008) Control of the risk of human toxoplasmosis transmitted by meat. International Journal for Parasitology 38: 1359-1370.
7. Meerburg BG, Riel, J. W. van, Cornelissen JB, Kijlstra A, Mul MF (2006) Cats and goat whey associated with Toxoplasma gondii infection in pigs. Vector-Borne and Zoonotic Diseases 6: 266-274.
8. Kijlstra A, Meerburg BG, Bos AP (2009) Food safety in free-range and organic livestock systems: risk management and responsibility. Journal of Food Protection 72: 2629-2637.

Appendix C. Additional search terms WP2

Database: MEDLINE 1950 to present
Search Strategy:
----

1 "Distribution of cysts and tachyzoites in calves and pregnant cows inoculated with Toxoplasma".ti. (1) **[missed reference]**

2 exp Sus scrofa/ (12022)

3 exp Sheep/ (102672)

4 exp Goats/ (25073)

5 exp Cattle/ (292207)

6 exp Chickens/ (97230)

7 exp Turkeys/ (8879)

8 exp Horses/ (56435)

9 exp Food/ (1041720)

10 exp *Birds/ (80713)

11 exp Antibodies/ (678838)

12 exp Immunoassay/ (427381)

13 exp Immunologic Tests/ (399494)

14 exp Immunoprecipitation/ (89536)

15 exp Polymerase Chain Reaction/ (364223)

16 exp Biological Assay/ (33334)

17 (bioassay$ or (bio adj assay$)).ti,ab. (30843)

18 exp Toxoplasma/gd, im, ip, ps, py [Growth & Development, Immunology, Isolation & Purification, Parasitology, Pathogenicity] (7462)

19 exp Toxoplasmosis/ (16545)

20 exp Swine Diseases/ (24147)

21 exp Cattle Diseases/ (59151)

22 exp Sheep Diseases/ (23452)

23 exp Bird Diseases/ (36041)

24 exp Horse Diseases/ (22746)

25 exp Goat Diseases/ (3778)

26 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 (1535962)

27 18 or 19 (18408)

28 11 or 12 or 13 or 14 or 15 or 16 or 17 (1592357)

29 20 or 21 or 22 or 23 or 24 or 25 (158455)

30 (26 or 29) and 27 and 28 (1020)

31 limit 30 to "review articles" (26)

32 30 not 31 (994)

33 limit 32 to (english or french or german) (940) **[2-33 old search strategy WP2]**

34 exp Sus scrofa/ (12022)

35 exp Sheep/ (102672)

36 exp Goats/ (25073)

37 exp Cattle/ (292207)

38 exp Chickens/ (97230)

39 exp Turkeys/ (8879)

40 exp Horses/ (56435)

41 exp Food/ (1041720)

42 exp *Birds/ (80713)

43 exp Toxoplasma/gd, im, ip, ps, py [Growth & Development, Immunology, Isolation & Purification, Parasitology, Pathogenicity] (7462)

44 exp Toxoplasmosis/ (16545)

45 exp Swine Diseases/ (24147)

46 exp Cattle Diseases/ (59151)

47 exp Sheep Diseases/ (23452)

48 exp Bird Diseases/ (36041)

49 exp Horse Diseases/ (22746)

50 exp Goat Diseases/ (3778)

51 exp Poultry Diseases/ (23352)

52 exp Poultry/ (122181)

53 exp *Toxoplasma/ (7310)

54 34 or 35 or 36 or 37 or 38 or 39 or 40 or 41 or 42 or 52 (1538554)

55 45 or 46 or 47 or 48 or 49 or 50 or 51 (158455)

56 43 or 44 or 53 (19762)

57 (54 or 55) and 56 (1749)

58 exp Risk Factors/ (554405)

59 exp *risk/ (23182)

60 exp seroepidemiologic studies/ (17406)

61 exp *epidemiologic studies/ (7347)

62 exp Risk Assessment/ (174394)

63 exp Food Contamination/ (52877)

64 exp Prevalence/ (187402)

65 risk$.ti. (270422)

66 57 and (58 or 59 or 60 or 61 or 62 or 63 or 64 or 65) (524)

67 limit 66 to yr="1994 -Current" (434)

68 limit 67 to (english or french or german) (418)

69 limit 68 to "review articles" (30)

70 68 not 69 (388) **[34-70 search strategy WP3]**

71 exp Lymph Nodes/ (69362)

72 (26 or 29) and 27 and (28 or 71) (1032)

73 72 not 30 (12) **[additional records when lymph nodes is added]**

74 (26 or 29) and 27 (1706)

75 1 and 74 (1) **[adding lymph nodes would retrieve missed reference]**

76 exp Tissues/ (1478055)

77 (26 or 29) and 27 and (28 or 76) (1084)

78 77 not (33 or 70) (143) **[additional records when tissues is added]**

79 limit 78 to "review articles" (28)

80 78 not 79 (115)

81 limit 80 to (english or french or german) (56)

82 1 and 81 (1) **[adding tissues would retrieve missed reference]**

83 exp musculoskeletal system/ or exp digestive system/ or exp respiratory system/ or exp urogenital system/ or exp endocrine system/ or exp cardiovascular system/ or exp nervous system/ or exp sense organs/ or exp tissues/ or exp "fluids and secretions"/ (6042023)

[FINAL choice: specific tissues selected as search terms]

84 (26 or 29) and 27 and (28 or 83) (1195)

85 84 not (33 or 70) (251)

86 limit 85 to "review articles" (39)

87 85 not 86 (212)

88 limit 87 to (english or french or german) (142) **[FINAL choice: additional records Medline]**

Appendix D. Overview anatomical distribution

Table D1. Pigs

	114 (Frazão-Teixeira, 2011)_MBio	231 (Fortier, 1990)_IHC	231 (Fortier, 1990)_MBio	523 (Bezerra, 2012)_MBio	523 (Bezerra, 2012)_PCR	523 (Bezerra, 2012)_IHC	527 (Bayarri, 2012)_MBio	538 (Shou, 2011)_PCR	555 (Verhelst, 2011)_MBio	555 (Verhelst, 2011)_qPCR	625 (Opsteegh, 2010)_MCqPCR	625 (Opsteegh, 2010)_MBio	740 (Belfort-Neto, 2007)_PCR	767 (Luis Garcia, 2006)_MBio	767 (Luis Garcia, 2006)_PCR	800 (Luis Garcia, 2005)_MBio	822 (Kringel 2004)_MBio	913 (Jungersen, 1999)_IHC	947 (Dubey, 1998)_MBio	999 (Dubey, 1996)_MBio	1023 (Dubey, 1994)_MBio	1029 (Pinckney, 1994)_MBio	1044 (Linday, 1993)_MBio	1110 (Dubey, 1988)_MBio	1110 (Dubey, 1988)_CBio	1151 (Prickett, 1985)_MBio	1158 (Dubey, 1984)_MBio	1224 (Chhabra, 1979)_MBio	1242 (Beverley, 1978)_MBio	1298 (Botros, 1973)_MBio	1336 (Shimizu, 1970)_MBio	1345 (Catar, 1969)_MBio	1371 (Juránková, 2014)_qPCR	1415 (de Cássia da Silva, 2010)_PCR	1465 (Katsube, 1968)_MBio	1527 (Work, 1968)_MBio	1538 (Wang, 2013)_MBio
brain	3/19	1/300	7/300	11/20	2/20	6/20			8/8	8/8	1/3	1/4		8/10	6/10	6/10	8/8		6/8	21/39	5/5	2/2	3/4	11/16		4/11	6/6	0/17	1/1	0/1	2/6	13/30	6/6				
spinal cord																											1/6										
heart	2/16	4/300	11/300						8/8	8/8	35/41	3/4			6/10					14/42	1/5	2/2	3/4	11/16	4/4	6/11	6/6			1/1	2/6		6/6				3/5
skeletal muscle*																								4/10					0/1								5/5
loin/tenderlion (longismus)*							0/25				30/41	1/4												2/7	1/4											1/1	
shoulder loin*							0/25																													1/1	
tongue				9/20	0/20	4/20	0/25				1/4		17/50		6/10		8/8		7/8	27/39	3/5	2/2	4/4	9/15	3/4		6/6										5/5
masseter muscle*															1/10																						
diaphragm		4/300	11/300								1/4		33/50		1/10				6/8					6/16	2/4	1/11	4/6	1/17			1/6	7/30			4/65		5/5
abdominal muscle*											0/4	0/4																									
thigh muscle*																											4/6										
thorax muscles/ribs*							2/25																	2/7	2/4						2/6					1/1	
scapular muscle*											0/4																										
limb muscle*																																				1/1	
left front limb*																																	3/6				
right front limb*																																	4/6				
left hind limb*																																	5/6				

	114 (Frazão-Teixeira, 2011)_MBio	231 (Fortier, 1990)_IHC	231 (Fortier, 1990)_MBio	523 (Bezerra, 2012)_MBio	523 (Bezerra, 2012)_PCR	523 (Bezerra, 2012)_IHC	527 (Bayarri, 2012)_MBio	538 (Shou, 2011)_PCR	555 (Verhelst, 2011)_MBio	555 (Verhelst, 2011)_qPCR	625 (Opsteegh, 2010)_MCqPCR	625 (Opsteegh, 2010)_MBio	740 (Belfort-Neto, 2007)_PCR	767 (Luis Garcia, 2006)_MBio	767 (Luis Garcia, 2006)_PCR	800 (Luis Garcia, 2005)_MBio	822 (Kringel 2004)_MBio	913 (Jungersen, 1999)_IHC	947 (Dubey, 1998)_MBio	999 (Dubey, 1996)_MBio	1023 (Dubey, 1994)_MBio	1029 (Pinckney, 1994)_MBio	1044 (Linday, 1993)_MBio	1110 (Dubey, 1988)_MBio	1110 (Dubey, 1988)_CBio	1151 (Prickett, 1985)_MBio	1158 (Dubey, 1984)_MBio	1224 (Chhabra, 1979)_MBio	1242 (Beverley, 1978)_MBio	1298 (Botros, 1973)_MBio	1336 (Shimizu, 1970)_MBio	1345 (Catar, 1969)_MBio	1371 (Juránková, 2014)_qPCR	1415 (de Cássia da Silva, 2010)_PCR	1465 (Katsube, 1968)_MBio	1527 (Work, 1968)_MBio	1538 (Wang, 2013)_MBio
right hind limb*																																	3/6				
bacon*																								1/7	2/4												
arm picnic*																								3/7	2/4												
boston butt*																								2/7	2/4												
gastrocnemius muscle*									4/8	3/8																											
ham*																						2/2	4/4	3/7	2/4												
dorsal muscle*											0/4																						5/6				
longissimus dorsi*									6/8	3/8																1/11											
neck muscle*																																				1/1	
muscle pool*														10/10		8/10																					
belly muscle*																																				1/1	
tail																								1/7													
liver								1/117										1/1						1/7	1/4	1/11	0/6			0/1	0/6		3/6				
kidneys																								1/7	1/4		2/6			0/1	0/6		3/6				
small intestine																		0/1									2/6										
intestine mucosa+submucosa																																		1/4			
intestine external muscle +serosa																																		0/4			
spleen																		1/1							0/4		0/6				0/6		0/6				
lungs																		1/1									0/6	0/17			0/6		6/6				
mesenteric lymph nodes																											0/6										

	114 (Frazão-Teixeira, 2011)_MBio	231 (Fortier, 1990)_IHC	231 (Fortier, 1990)_MBio	523 (Bezerra, 2012)_MBio	523 (Bezerra, 2012)_PCR	523 (Bezerra, 2012)_IHC	527 (Bayarri, 2012)_MBio	538 (Shou, 2011)_PCR	555 (Verhelst, 2011)_MBio	555 (Verhelst, 2011)_qPCR	625 (Opsteegh, 2010)_MCqPCR	625 (Opsteegh, 2010)_MBio	740 (Belfort-Neto, 2007)_PCR	767 (Luis Garcia, 2006)_MBio	767 (Luis Garcia, 2006)_PCR	800 (Luis Garcia, 2005)_MBio	822 (Kringel 2004)_MBio	913 (Jungersen, 1999)_IHC	947 (Dubey, 1998)_MBio	999 (Dubey, 1996)_MBio	1023 (Dubey, 1994)_MBio	1029 (Pinckney, 1994)_MBio	1044 (Linday, 1993)_MBio	1110 (Dubey, 1988)_MBio	1110 (Dubey, 1988)_CBio	1151 (Prickett, 1985)_MBio	1158 (Dubey, 1984)_MBio	1224 (Chhabra, 1979)_MBio	1242 (Beverley, 1978)_MBio	1298 (Botros, 1973)_MBio	1336 (Shimizu, 1970)_MBio	1345 (Catar, 1969)_MBio	1371 (Juránková, 2014)_qPCR	1415 (de Cássia da Silva, 2010)_PCR	1465 (Katsube, 1968)_MBio	1527 (Work, 1968)_MBio	1538 (Wang, 2013)_MBio
prescapular lymph node																											0/6										
hilar lymph node								52/225																													
bronchial lymph nodes																		1/1								0/11											
intestinal lnn																		1/1													0/6						
eye(s)																											0/6								1/8		
chorio-retinal coat																																			2/8		
pancreas																															0/6						
salivary gland																											0/6										
Meat/muscle combined*							2/25		6/8	3/8	30/41	1/4		10/10	1/10	8/10						2/2	4/4	10/16	2/4	1/11	4/6		0/1		2/6		6/6			1/1	5/5

Table D2. Cattle

	429 (Wyss, 2000)_PCR	429 (Wyss, 2000)_IHC	639 (Lima Santos, 2010)_PCR	919 (Esteban-Redondo, 1999)_MBio	919 (Esteban-Redondo, 1999)_Histo	919 (Esteban-Redondo, 1999)_PCR	1017 (Arias, 1994)_MBio	1046 (Dubey, 1993)_CBio	1046 (Dubey, 1993)_MBio	1046 (Dubey, 1993)_Histo	1066 (Dubey, 1992)_CBio	1066 (Dubey, 1992)_MBio	1237 (Munday, 1978)_MBio	1251 (Costa, 1977)_MBio	1387 (Dubey, 1983)_MBio_calves	1387 (Dubey, 1983)_ CBio_calves	1387 (Dubey, 1983)_MBio_cows	1387 (Dubey, 1983)_CBio_cows	1398 (Beverley, 1977)_ MBio
brain	5/350	0/9	2/100	1/10	0/5	0/10		1/4	0/4	0/4	0/1		0/4	0/5	1/4		0/6		0/9
spinal cord					0/5				0/4	0/4	0/1				1/4		0/6		
heart			0/100	0/10	0/5	0/10	0/10	3/4	0/4	0/4	0/1			0/5	0/4	3/5	0/6	1/3	0/9
skeletal muscle*																			
psoas muscle*				0/10	0/5	0/10													
gracilis muscle*				0/10	0/5	0/10													
loin/tenderloin (longissimus)*							1/10	0/4	0/4	0/4									
tongue								2/4	0/4	0/4	0/1				2/4	3/3	0/6		
masseter muscle*	4/350	0/9																	
diaphragm												0/1	2/4	2/5	0/4	1/2	0/6		
thigh muscle*							3/10								2/4	3/6	0/6	2/6	
thorax muscles/ribs*							0/10	1/4	0/4	0/4	0/1								
muscles from limbs and carcass*											0/1								
muscle "top round"*							1/10												
muscle "top round steak"*							2/10												

	429 (Wyss, 2000)_PCR	429 (Wyss, 2000)_IHC	639 (Lima Santos, 2010)_PCR	919 (Esteban-Redondo, 1999)_MBio	919 (Esteban-Redondo, 1999)_Histo	919 (Esteban-Redondo, 1999)_PCR	1017 (Arias, 1994)_MBio	1046 (Dubey, 1993)_CBio	1046 (Dubey, 1993)_MBio	1046 (Dubey, 1993)_Histo	1066 (Dubey, 1992)_CBio	1066 (Dubey, 1992)_MBio	1237 (Munday, 1978)_MBio	1251 (Costa, 1977)_MBio	1387 (Dubey, 1983)_MBio_calves	1387 (Dubey, 1983)_ CBio_calves	1387 (Dubey, 1983)_MBio_cows	1387 (Dubey, 1983)_CBio_cows	1398 (Beverley, 1977)_ MBio
muscle "stirk steak"*							3/10												
muscle "brisket"*							1/10												
muscle "roast" (semimembranosus and semitendinosus)*								1/4	0/4	0/4									
unspecified muscle*														2/5					0/9
liver					0/5		5/10	2/4	0/4	0/4	0/1			0/5	3/4	3/5	0/6	2/5	
kidneys					0/5			0/4	0/4	0/4	0/1			0/5	1/4	1/2	0/6	0/3	
small intestine								1/2	0/4	0/4		1/1		1/5	2/4		1/6		
spleen								0/1	0/4	0/4		0/1		1/5	0/4		0/6		
lungs					0/5		0/10		0/4	0/4		0/1		2/5	0/4		0/6		
mesenteric lymph nodes					0/5				0/4	0/4		0/1			0/4		1/6		
prescapular lymph node															0/4		0/6		
unspecified lymph nodes														4/5					3/9
eye(s)												0/1	0/4	2/5	0/4		0/6		
pancreas															0/4		0/6		
adrenal glands															0/4		0/6		
thyroid glands															0/4		0/6		

	429 (Wyss, 2000)_PCR	429 (Wyss, 2000)_IHC	639 (Lima Santos, 2010)_PCR	919 (Esteban-Redondo, 1999)_MBio	919 (Esteban-Redondo, 1999)_Histo	919 (Esteban-Redondo, 1999)_PCR	1017 (Arias, 1994)_MBio	1046 (Dubey, 1993)_CBio	1046 (Dubey, 1993)_MBio	1046 (Dubey, 1993)_Histo	1066 (Dubey, 1992)_CBio	1066 (Dubey, 1992)_MBio	1237 (Munday, 1978)_MBio	1251 (Costa, 1977)_MBio	1387 (Dubey, 1983)_MBio_calves	1387 (Dubey, 1983)_ CBio_calves	1387 (Dubey, 1983)_MBio_cows	1387 (Dubey, 1983)_CBio_cows	1398 (Beverley, 1977)_ MBio
salivary gland															0/4		0/6		
thymus															0/4		0/6		
uterus												0/1							
placenta																			
testicle														2/5	0/4				
blood														5/5				0/6	
colostrum																	0/6		
Meat/muscle combined*	4/350	0/9		0/10	0/5	0/10	11/70	1/4	0/4	0/4	0/1			2/5	1/4	3/6	0/6	2/6	0/9

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Table D3. Sheep

	429 (Wyss, 2000)_ PCR	429 (Wyss, 2000)_IHC	457 (Glor, 2013)_qPCR Exp	457 (Glor, 2013)_qPCR nat	467 (Silva, 2013)_IHC	883 (Vieira da Silva, 2001)_MBio	883 (Vieira da Silva, 2001)_PCR	919 (Esteban-Redondo, 1999)_MBio	919 (Esteban-Redondo, 1999)_PCR	941 (Esteban-Redondo, 1998)_PCR	941 (Esteban-Redondo, 1998)_Histo	1101 (Dubey, 1989)_MBio	1281 (Hartley, 1974)_MBio	1315 (Punke, 1971)_MBio	1370 (Juránková, 2013)_PCR	1432 (Dubey, 1980)_MBio	1646 (Gutierrez, 2010)
heart					6/26			7/8	4/8	6/12	0/12	3/8		3/101	1/6	2/5	
brain	5/150	0/9	4/6	6/96	3/26	31/39	8/39	5/8	6/8	5/12	0/12		87%	2/101	6/6	4/5	
spinal cord																2/5	
skeletal muscle*																5/5	
psoas muscle*								4/8	0/8	0/12	0/12						
gracilis muscle*								2/8	0/8	1/12	0/12						
tongue												7/8					
masseter muscle*	7/150	0/9		7/96													
diaphragm						30/39	9/39						55%			4/5	
thorax muscles/ribs*												8/8					
front limb muscles*															2/6		
hind limb muscles*			5/6									8/8			1/6		
dorsal muscle*															1/6		
neck muscle*																	
liver					10/26						0/12				0/6	1/5	

	429 (Wyss, 2000)_ PCR	429 (Wyss, 2000)_IHC	457 (Glor, 2013)_qPCR Exp	457 (Glor, 2013)_qPCR nat	467 (Silva, 2013)_IHC	883 (Vieira da Silva, 2001)_MBio	883 (Vieira da Silva, 2001)_PCR	919 (Esteban-Redondo, 1999)_MBio	919 (Esteban-Redondo, 1999)_PCR	941 (Esteban-Redondo, 1998)_PCR	941 (Esteban-Redondo, 1998)_Histo	1101 (Dubey, 1989)_MBio	1281 (Hartley, 1974)_MBio	1315 (Punke, 1971)_MBio	1370 (Juránková, 2013)_PCR	1432 (Dubey, 1980)_MBio	1646 (Gutierrez, 2010)
kidneys											0/12				0/6	1/5	
small intestine																1/4	
spleen															0/6	2/5	2/18
pancreas																1/5	
lungs											0/12				3/6	1/4	
mesenteric lnn											0/12					1/5	
uterine lnn																	1/18
cervical lnn																0/5	
eye(s)																0/5	0/18
adrenal glands																1/5	
salivary gland																0/5	
mammary glands																1/4	
uterus																1/5	
blood																	0/18
Meat/muscle combined*	7/150	0/9	5/6	7/96				5/8	0/8	1/12	0/12	8/8			2/6	5/5	

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Table D4. Goats

	148 (Silva, 2009)_PCR	460 (Jurankova, 2013)_PCR	666 (Ragozo, 2009)_MBio	892 (Nishi, 2001)_MBio	1423 (Dubey, 1980)_MBio_Exp	1423 (Dubey, 1980)_MBio_Nat	1424 (Dubey, 1980)_MBio	1433 (Dubey, 1980)_MBio	1451 (Hartley, 1982)_Histo	1482 (Kazacos, 1983)_Histo
heart	1/102	12/12		6/6	3/6	6/9		5/6		
brain	4/102	12/12		5/6	3/6	3/10		6/6		
pool of brain and heart			8/26							
spinal cord							0/1	5/6		
spinal fluid							0/1			
skeletal muscle*				6/6	5/6	10/10	1/1	5/6		
tongue	3/102									
diaphragm					3/6	6/9		5/5		
pool of masseter muscle and diaphragm*			7/26							
muscles front limbs*		12/12								
muscles hind limbs*		12/12								
dorsal muscle*		12/12								
liver		10/12		4/6	5/6	3/10		3/6	1/1	1/1
kidneys		11/12		4/6	5/6	3/10		5/6	1/1	1/1
small intestine								4/4		

	148 (Silva, 2009)_PCR	460 (Jurankova, 2013)_PCR	666 (Ragozo, 2009)_MBio	892 (Nishi, 2001)_MBio	1423 (Dubey, 1980)_MBio_Exp	1423 (Dubey, 1980)_MBio_Nat	1424 (Dubey, 1980)_MBio	1433 (Dubey, 1980)_MBio	1451 (Hartley, 1982)_Histo	1482 (Kazacos, 1983)_Histo
spleen		12/12						2/6		1/1
lungs		12/12						2/6		
mesenteric lymph nodes								4/6		
cervical lymph nodes								3/4		
unspecified lymph nodes				3/6						
eye(s)								1/5		
pancreas								4/5		1/1
adrenal glands								0/5		
salivary gland								3/3		
thymus								2/4		
urinary bladder								0/1		
testicle								0/1		
mammary glands								2/2		
blood								0/4		
Meat/muscle combined*		12/12	7/26	6/6	5/6	10/10	1/1	5/6		

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Table D5. Chicken

	32 (Chumpolbanchorn, 2013)_PCR	129 (Asgari, 2009)_PCR	266 (Goncalvesa, 2012)_PCR	563 (Aigner, 2010)_PCR	595 (Yan, 2010)_PCR	705 (Dubey, 2007)_MBio	756 (Dubey, 2005)_MBio	778 (Dubey, 2006)_MBio	779 (Dubey, 2006)_MBio	782 (Dubey, 2005)_MBio	783 (Dubey, 2005)_MBio	795 (Dubey, 2005)_MBio	805 (Dubey, 2005)_MBio	815 (Dubey, 2004)_MBio	965 (Kaneto, 1997)_MBio	1037 (Dubey, 1993)_MBio	1364 (Boch, 1966)_MBio	1460 (Jacobs, 1966)_MBio_pools	1460 (Jacobs, 1966)_MBio_ind	1460 (Jacobs, 1966)_MBio_expinf	1713 (Deyab, 2005)_Histo
heart		25/29	15/100	21/26	5/12	11/11	8/19	10/14	17/33	16/22	10/13	33/43	9/11	10/13	4/21	3/5	18/27				2/28
brain	10/27	16/29	22/100	22/26	4/12	5/11	3/19	4/14	16/33	12/22	7/13	24/43	4/15	0/13	12/21	5/5	16/27		0/180	14/26	10/28
pool of heart and brain													6/88								
bone marrow																	1/21				
skeletal muscle*															1/21		4/27				6/28
pectoral muscle*							4/19			1/22	3/13	2/43	3/11	0/13		0/5				9/14	
limb muscle*						8/11		5/10								2/5			1/180	10/14	
pool of brain, heart and leg muscle								5/5													
pool of brain, heart and pectoral muscle											1/3	1/10	1/14								
liver		27/29			2/12										2/21	0/5	5/26				4/28
kidneys															2/21		4/26			4/10	
proventriculus															3/21						
ventriculus (gizzard)																				9/12	
intestine															2/21		0/26			9/14	
spleen	12/23				4/12										5/21		3/27				

	32 (Chumpolbanchorn, 2013)_PCR	129 (Asgari, 2009)_PCR	266 (Goncalvesa, 2012)_PCR	563 (Aigner, 2010)_PCR	595 (Yan, 2010)_PCR	705 (Dubey, 2007)_MBio	756 (Dubey, 2005)_MBio	778 (Dubey, 2006)_MBio	779 (Dubey, 2006)_MBio	782 (Dubey, 2005)_MBio	783 (Dubey, 2005)_MBio	795 (Dubey, 2005)_MBio	805 (Dubey, 2005)_MBio	815 (Dubey, 2004)_MBio	965 (Kaneto, 1997)_MBio	1037 (Dubey, 1993)_MBio	1364 (Boch, 1966)_MBio	1460 (Jacobs, 1966)_MBio_pools	1460 (Jacobs, 1966)_MBio_ind	1460 (Jacobs, 1966)_MBio_expinf	1713 (Deyab, 2005)_Histo
lungs					5/12										1/21		5/27				0/28
eye(s) - retina					4/12										5/21		4/21				
pancreas															5/21						
ovaries																	6/22	5/24	4/180	9/14	
ovary duct																	4/22	6/24		11/14	
pool of ovaries and oviducts																		4/38			
eggs																			0/180	1/327	
testicle																	1/5				
Meat/muscle combined*						8/11	4/19	5/10		1/22	3/13	2/43	3/11	0/13	1/21	2/5	4/27		1/180	11/14	6/28

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Table D6. Turkeys

	30 (Bangoura, 2013)_PCR	311 (Sedlak, 2000)_MBio	1034 (Dubey, 1993)_MBio	1454 (Howerth, 1985)_Histo	1596 (Zoller, 2013)_PCR
heart	8/36	5/5	5/5		7/36
brain	17/36	3/5	0/5	1/1	3/36
limb muscle (drum stick)*	8/36	2/5	4/5		8/36
thigh muscle*	9/36				7/36
breast muscle*	3/36		2/5		10/36
pool of heart, breast muscle and limb muscle			6/6		
liver	2/36	2/5	0/5	1/1	13/36
kidneys	2/36			1/1	6/36
proventriculus	6/36			1/1	3/36
ventriculus (gizzard)	6/36			0/1	3/21
intestine	6/36				6/36
colon				1/1	
spleen	3/36	1/5		1/1	2/36
lungs	5/36			1/1	2/36
pancreas	1/36				6/36
adrenal glands				1/1	
oesophagus				1/1	
ovaries				0/1	

	30 (Bangoura, 2013)_PCR	311 (Sedlak, 2000)_MBio	1034 (Dubey, 1993)_MBio	1454 (Howerth, 1985)_Histo	1596 (Zoller, 2013)_PCR
testicle	2/36				2/27
blood		1/5			
Meat/muscle combined*	17/36	2/5	4/5		

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Table D7. Horses

	1146 (Dubey, 1985)_MBio	1213 (Al Khalidi, 1980)_MBio	1373 (Altan, 1977)_MBio
heart	3/13	4/9	1/4
brain	1/13	2/4	0/4
cerebrum		0/5	
cerebellum		0/5	
spinal cord	1/13	2/9	
skeletal muscle		1/9	
tongue	4/13		
diaphragm	0/13	1/9	
thigh muscle	1/13		
liver	0/13	1/9	0/4
kidneys	1/13	1/9	
small intestine	4/13		
spleen	0/13	0/9	0/4
lungs	1/13	0/9	0/4
mesenteric lymph nodes	0/13	0/9	0/4
eye(s)	0/13	0/9	
pancreas		0/9	

	1146 (Dubey, 1985)_MBio	1213 (Al Khalidi, 1980)_MBio	1373 (Altan, 1977)_MBio
stomach		0/9	
adrenal glands		0/9	
Meat/muscle combined*	1/13	1/9	

* all meat cuts and muscle tissues shaded in green are included in the meat/muscle combined category.

Appendix E. Farm risk factors and *T. gondii* infection

Supplementary Table S1: Results on risk factor analyses in pigs

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Definitive host related				
Access of cats	Cat access to sows (R,37,47), Cat access to swine facilities (R,56), Cats had access to animal housing at times prior to the interview (R,38), Cats had access to the fodder-storage room at times prior to interview (R,38), Cats have access to animal housing at the date of interview (R,38), Cats have access to the fodder-storage room at the date of interview (R,38)		7	1008, 1380
Cat-Toxoplasma-Vaccination	Vaccination/repeated vaccination of juvenile and adult cats with Toxoplasma T-263 bradyzoite live vaccine resulted in decreased seroprevalence compared to prevalence prior to intervention (R,35)	1		914
Contact with cat feces	Contact with cat feces assumed possible (R,56)		1	749
High cat density	High cat-density (two or more cats were present in 10,000 square meters of pig farms) (R,30)		1	392
High frequency of cats exposure	Pigs with high frequency of cat exposure have a higher risk of being seropositive (R,44)		1	578

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Increasing number of cats	Actual number of cats present on farm: 1-2 (R,38), Actual number of cats present on farm: 3-4 (R,38), Actual number of cats present on farm: 5-15 (R,38), High cat number on farm: higher seroprevalence vs Low cat number on farm (R,46), More than 3 cats present on farm (R,56), Number of cats trapped (R,56), Number of juvenile cats on the farm (R,56), Number of juvenile cats trapped (R,56), Number of seropositive cats (R,56),Number of seropositive juvenile cats trapped (R,56), Previous number of cats present on farm: 1-2 (R,38), Previous number of cats present on farm: 3-4 (R,38),Previous number of cats present on farm: 5-15 (R,38)		17	38, 749, 1008, 1380
Presence of cats	At date of sampling cats were on farm (R,38), Cats presence (R,64), Cats' presence (R,76), Cats present (R,64), Presence of cats (R,70), There are cats on farm (R,38), Use of cats to fight rodents (R,38)		7	604, 621, 1011, 1380
Presence of *Toxoplasma gondii* oocysts	Oocysts detected on farm (cat faeces, pig feed, soil) (R,56)		4	1008
Feed-related				
Feed contamination possible	Feeding is manually (R,38), Manual feeder vs Automatic feeder (P,60), Roughage not covered (R,56), Silo is open (R,38), Storage of fodder in open containers (R,38), Type of feeder manual vs Type of feeder automatic (R,46)	1	5	38, 749, 1380
Feed contamination unlikely	Feeding is automated (P,38), Silo is closed (P,38), Storage of fodder in closed containers (R,38), Storage of fodder is generally closed (P,38)	3	1	1380
Feeding goat whey	Goat whey is fed to pigs (R,56)		2	749

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Type of feed - dry	Dry feeding (R,38)		1	1380
Type of feed - fluid	Fluid feeding (R,38)	1		1380
Type of feed storage - silo	Food storage in a silo vs Food storage in a warehouse (R,46)		1	38
Housing related				
Floor other than grid, full slatted floor, partially slatted floor or with straw bedding	Floor other than grid, full slatted floor, partially slatted floor or with straw bedding (R,38)		1	1380
Perforated or slatted floor	There are full slatted floors on farm (R,38)	1		1380
Straw bedding	Straw-bedding is used on farm (R,38)		1	1380
Housing-in/out, cleaning and disinfection				
All-in-all-out	All-in-all-out (R,38)	1		1380
Increasing time of stable without animals	Stable is empty prior to entry of new animals > 4-240 days vs Stable is empty prior to entry of new animals 0 - 1 days (P,38)	1		1380
Manual cleaning	Cleaning method manual vs Cleaning method semi-automatic (R,58), Manual cleaning method in fattening vs Semi-automatic cleaning in fattening (R,76)		2	404, 604
No all-in-all-out	All-in-all-out housing is absent (R,83), Animals are brought-in/taken-out continuously (R,38), No all in/all out in fattening (R, 76)		3	551, 604, 1380
No disinfection	Cleaning by only removing the manure (R,38), No disinfection protocol in fattening (R,76)		2	1380

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
No frequent disinfection	Pigs from farms which disinfect only one/week but not multiple/week had a higher risk of being seropositive (R,44)		1	578
Only mechanical cleaning, no disinfection	Cleaning only mechanical vs Cleaning mechanical and chemical disinfection (R,83)		1	551
Level of confinement and management intensity				
Outside stable access	Animals have access to outside pens vs Animals in stable only (R,55), [Extensive Antibiotic-free pig production vs conventional intensive production (R,22)], Farms keeping swine in huts (R,38), Farms with animal-friendly systems (organic & free-range) have a higher seroprevalence than conventional farms (R,54), Outdoor facilities for sows (R,70), Pigs allowed to scavenge once/day had a higher risk of being positive compared to those allowed to scavenge multiple/per day (R,44), Pigs reared in partially open confinements had a higher risk of being seropositive compared to those from closed confinements (R,44), Sows kept in partial confinement vs Sows kept in total confinement (R,64)		8 [1]	194, 578, 621, 721, 764, 1011, 1380

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Pastured	Pigs reared free-range vs Pigs reared intensive (R,46), Pigs reared free-range vs Pigs reared intensively (R,30), Sows are entirely pastured or kept in partial confinement vs Sows are entirely pastured or kept in partial confinement (R,64), Sows are entirely pastured or kept in partial confinement vs Sows kept in total confinement (R,64), Sows are entirely pastured vs Sows are kept in total confinement (R,64), Sows are entirely pastured vs Sows kept in partial confinement (P,64)	1	6	474, 706, 1011
Rodent related				
No rodent control	No rodent control (R,76), No rodenticides used (R,58), Rodent control (R,64)		4	404, 604
Presence of rodents	No presence of rodents: higher seroprevalence vs Presence of rodents (P,37)	1		38
Rodent control	Duration of rodent control (P,37), Farm uses traps, bait, poison, exterminator against rodents (P,74), Use of chemicals, traps or destruction of habitats against rodents vs Cats and rodent-proof containers (R,64)	2	1	158, 630, 1011
Seropositivity of rodents	House mouse Toxoplasma gondii seroprevalence (R,56), Toxoplasma seroprevalence in house mice (R,57)		3	1008
Related to water provided to animals				
Contamination of water point possible	Drinking water is provided in a trough (R,38)		1	1380

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Surface water	Pigs provided surface water had a higher risk of being seropositive vs Pigs provided water from tap or well (R,44), Water supply from shallow wells vs Municipal water supply (R,58)		2	404, 578
Biosecurity related				
Adequate remove of dead animals	No exposure to dead swine carcass (P,83)	1		551
Low level of biosecurity	No insect control (R,76), No-bird proof nets (R,76), Pigs from farms at which mosquitoes and flies are present have a higher risk of being seropositive (R,44),		3	578, 604
Not adequate remove of dead animals	Farm buries dead weaned pigs off site (R,74), Farm composts dead preweaned pigs on site (R,74)		2	578, 630
Low level of personnel hygiene	A hygiene sluice is not always used (R,38), Hands are not washed prior to entering the stable (R,38), No boots are available (R,38), No hygiene sluice is available (R,38), No overall is available (R,38), No personal protective clothing available on farm (R,38), Protective clothing is not changed in a hygiene sluice (R,38)		7	1380
Climate				
Humidity high	Mean monthly relative humidity (%) 64–66 vs Mean monthly relative humidity (%) <64 (R,76)		1	604
Rainfall high	Annual cumulative rainfall (mm) 396–507 vs Annual cumulative rainfall (mm) <396 (R,76)		1	604

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Temperature high	Mean monthly temperatures (°C) >15,4 vs Mean monthly temperatures (°C) <13,7 (R,76); Mean monthly temperatures (°C) 13,7-15,4 vs Mean monthly temperatures (°C) <13,7 (R,76)		2	604
Season related				
Slaughter autumn/winter	Slaughter during autumn/winter (R,29)		1	789
Extent of specialization				
Low level of specialization	[Backyard pigs vs Sows or fattening pigs (R,25)], Dogs' presence (R,76), There are cattle on farm (R,38), There are ruminants on farm (R,38), There is other livestock on farm (R,38), Farms using no sulfonamides (R,44)		6 [1]	443, 578, 604, 1380
High level of specialization	No other species reared (P,58), Poultry absent (P,58)	2		404
Purpose of livestock				
Farming type: Not-Feeder-to-finish	Farm type - Farrow-to-finish vs Finishing type farming (R,58), Farm type: Breeding/piglet production including also weanling-to-feeder and/or finishing (R,38), Farming type: Farrow-to-finish vs Farming type: Farrow-to-weaning (R,76), Farming type: Farrrow to finish vs Farming type: Finishing (R,58), Farm type: Piglet production vs Farm type: Farrow to finish (R,71), Farm type: Piglet production vs Farm type: Pedigree breeding (R,71), Farrow to finish: higher seroprevalence than Feeder-to-finish (R,46)		7	404, 1380, 812, 38, 604

	Details on variables (Direction of effect[a] Quality score[b]) [Variable from a study of poor quality]	Number of studies reporting a… [Number of studies of poor quality reporting a…]		References
Categories of reported risk factors		**protective effect**	**risk effect**	
Farming type: Feeder-finish	Farm type: Finishing (R,55,73), Farm type: Feeder-to-finish (P,38)	1	2	764, 1380
Potential Toxoplasma effects				
Mortality in weaning	Mortality in weaning (>2%) (R,70)		1	621
Reproductive disorders	Farms with reproductive disorders (R,71)		1	812
Potentially size related				
Overall pig tested by farm	Overall pig tested by farm ((R,58)		1	404
Related to geographical localisation				
Farm located at hills	Pigs from farms located at hills vs Pigs coming from farms located on plains (R,44)		1	578
Low altitude	Farm's location <= 200 m above sea level (R,58)		1	404
Interactions				
Pigs reared on a farm: "Farrow-to finish" and "No rodent control" (R,68)			1	404
Pigs reared on a farm: "Farrow-to finish", "Manual cleaning", "No rodent control" farm (R,68)			1	404
Pigs reared on a farm: "Size <= 50", "located <= 200 m above sea level" farm (R,68)			1	404
There are no cats and sows are entirely pastured or kept in partial confinement vs Pasture and cats present (R,64)			1	1011
Other				
	No cannibalism, higher seroprevalence vs Cannibalism (R,46) Sample derived from a pig kept for pleasure (R,38) Sample derived from a pig of unknown age category (R,38)		3	38, 1380

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S2: Results on risk factor analyses in cattle

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Definitive host related				
Presence of cats	Presence of cats (R,74)		1	405
Related to geographical localization				
Farm not isolated	Neighbourhood index (isolated, one neighbour, two or more neighbours) (P,74)	1		405
Interactions				
Presence cats : Age	Presence cats : Age (P,74)	1		405
Neighbourhood index : Herd size	Neighbourhood index : Herd size (R,74)		1	405
Level of confinement				
Outside stable access	Cattle have access to outside pens vs Cattle are in stable only (P,55,69)	2		764
Related to water provided to animals				
Water point on pasture	Water point on pasture (R,74)		1	405
Animal density related				
Low cattle density	Cattle from herds with low cattle density have a higher risk of being seropositive (R,30)		1	463

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S3: Results on risk factor analyses in small-ruminants

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Definitive host related				
Access of cats	[Access of cats to goat feeding (G,R,26)], Access of stray cats to animals' water (S,R,51), Access of stray cats to animals' water (S,R,59), Cats have access to pantry vs Cats have access to pasture (S,R,33)		4 [1]	390, 503, 745
Contact with felines	Contact with felines (S,R,42)		1	636
No presence of wild felids	No presence of wild felids (S,R,50)		1	440
Presence of cats	Cats are present in the flock (SG,R,40), Cats on farm (S,R,47) Presence of cats (S,R,30), Presence of cats (SG,R,50), Presence of cats (SG,R,55), [Presence of cats near livestock (G,R,26)]		6 [1]	503, 531, 738, 993
Young cats presence	Young cat observed daily in sheep house (S,R,47), Young cat observed daily in sheep house (S,R,55)		2	945
Feed-related				
Concentrate feeding	Feeding of concentrate (SG,R,51)		2	471
Feed contamination possible	Manger and trough, pasture vs Manger, manger and trough (G,R,45)		1	1386

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Mineral supplementation	Mineral supplementation (S,R,42), Mineral supplementation by common salt vs Mineral supplementation by mineral salt (S,R,33)		2	636 745
Type of roughage	Feeding hay vs Feeding on pasture/Feeding fresh bulk feed (S,R,42)		1	636
Related to water provided to animals				
Public supply water	Tap water vs Water from river, well, lake, pond ("Mixed") (S,R,50), Tap water vs Water from river, pond, well, lake, pond ("Mixed") (S,R,73), Water from the public supply (SG,R,51)		3	440, 471
Surface and public water	Public and/or well, lake, stream vs Public water distribution (G,R,45)		1	1386
Surface water	River as source of water vs Water from river, well, lake, pond ("Mixed") (S,R,73), Use of surface water sources vs Use of water from wells (S,R,47), Water from dam, reservoir, spring vs Water from mine, artesian well (S,R,33), Water from pond, well, lake ("Stagnant") vs Water from river, well, lake, pond ("Mixed") (S,R,50), Water from river vs Water from river, well, lake, pond ("Mixed") (S,R,50)		5	440, 738, 745
Housing related				

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Timber construction	Timber construction of sheep house (S,P,47), Timber construction of sheep house (S,P,55)	2		945
Perforated or slatted floor	Perforated metal floor in sheep house (S,P,47), Perforated metal floor in sheep house (S,P,55)	2		945
Level of confinement and management intensity				
Lambing not in-house	Sheep kept in paddocks or parks during lambing vs Sheep in housing during lambing (S,R,47)		1	1622
Semi-extensively managed	Atypical grazing (S,R,55), Atypical grazing strategies (S,R,47), Management type: semi-extensive (S,R,50)		3	440, 945
Intensively managed	Intensive management (SG,R,50)		1	993
Higher level of management intensity	Agro-pastoral farming system vs Pastoral farming system (S,R,50), Management type intensive vs Management type extensive (SG,R,51), Management type semi-intensive vs Management type extensive (SG,P,51), Sedentary farming system vs Pastoral farming system (S,R,50), [Under intensive management conditions sheep had a higher risk of being seropositive (S,R,20)]	1	5 [1]	440, 471, 697

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Lower level of management intensity	Access to outside pens vs Stable (G,R,45), Access to pasture vs Stable (G,R,45), Extensive production system vs Semi-extensive production system (S,R,42), [Semi-intensive management vs Intensive management (G,R,26)], Sheep from extensive farms vs Sheep from intensive and semi-intensive farms (S,P,33)	1	5 [1]	503, 636, 745, 1386
Rodent related				
Presence of rodents	Presence of rodents in the food room (S,R,33)		1	745
Rodent control	Use of mouse poison (S,R,55)		1	945
Biosecurity related				
High level of biosecurity	Sheep from farms with a boundary to a single other farm vs Sheep from farms with boundaries to multiple other farms (S,P,47), Sheep have no contact with sheep from other farms vs Sheep share pasture with sheep from other farms (S,P,47)	2		1622
Low level of biosecurity	Aborted foetuses left on the ground vs Aborted foetuses fed to dogs (SG,R,55), Method of disposal aborted foetuses: Fetuses are left on the ground vs Fetuses are given to dogs (SG,R,40), Animals coming from outside source vs Animals born on farm (G,P,45), Animals coming from outside source vs Animals born on farm	2	2	531, 1386

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
	(G,P,49),			
Climate				
Higher humidity and temperature	[Animals reared at semi-warm humid climate had a higher prevalence than animals kept in other climates (Semi-warm sub-humid, Temperate sub-humid) (G,R,24)]		[1]	464
Extent of specialization				
Low level of specialization	Animals used for dairy, meat production and breeding vs Animals only used for dairy production (G,R,49), [Mixed exploration (dairy and meat) vs Meat exploration (G,26)], Cattle on premises (S,R,71), [Goats reared in backyards vs Goats reared in herds (G,R,26)], Mixed breed (S,R,43), [Mixed breed vs Pure breed (G,R,22)], No Anthelmintic treatment (SG,P,35), Presence of cattle on holding (S,R,77), Use of animals for dairy, meat production, breeding vs Use only for dairy (G,R,45)	1	5 [3]	25, 93, 444, 500, 503, 541, 1386

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
High level of specialization	Exclusively goats are present vs Also fowl/pigs/dogs/cats on farm (G,R,45), Goats are the only farm animal vs Also fowl/pigs/dogs/cats on farm (G,R,49), Grazing with the same species vs Grazing with sheep, goats, cattle, pigs, horse (SG,R,35)		3	25, 1386
Purpose of livestock				
Use of individual animal	Animal used for meat production (SG,R,50),		2	993
Potential Toxoplasma effects				
Abortion	Abortion occurred in the 3rd trimester of gestation vs Abortion occurred in the 1st/2nd trimester of gestation (SG,R,40), Occurrence of abortion outbreaks (SG,R,50)		2	531, 993
Mortality	Mortality was noticed within 24 h after abortion (SG,R,40)		1	531
Neurological problems	Neurological problems in lambs (S,R,33)		1	745
Potentially farm/flock size related				
Potentially related to large size	Number of breeding ewes per flock/10 (S,R,77)		1	541
Potentially related to small size	Absence of replacement animals in preceding year (SG,R,50)		1	993

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	Number of studies reporting a… [Number of studies of poor quality reporting a…]		References
Categories of reported risk factors		**protective effect**	**risk effect**	
Geography related				
Farm not isolated	Proximity to other farms (< 500 m) (SG,P,50)	1		993
High altitude	Altitude >500 m asl [d] vs Altitude 1-100 m (S,R,55), Altitude 251 - 500 m asl vs Altitude 1 - 100 m asl (S,R,55), Altitude 251 - 500 m asl vs Altitude 1 -100 m asl (S,R,47), Flock located > 2300 m above sea level vs Flock located < 1500 m asl (S,R,73), Flock located > 2300 m asl Flock located < 1500 m asl (S,R,50), Flock located 1500 – 2300 m asl vs Flock located < 1500 m asl (S,R,73), Flock located 1500 – 2300 m asl vs Flock located < 1500 m asl (S,R,50)		7	440, 945
Low altitude	Semi-mountainous farm location (200–700 m asl) vs Mountainous farm location (>700 m asl) (SG,R,35)		1	25
Mountainous grazing land	Mountainous area as grazing land vs Plain and mountainous area as grazing land (S,R,50)		1	440
Plain grazing land	Plain area as grazing land vs Plain and mountainous area as grazing land (S,R,50)		1	440
Type of soil	Black soil at farm or pasture (S,R,47)		1	945
Land cover				
Forest	Land cover: Forest vs Land cover: Savanna (SG,R,35)		1	25
Urban/crop	Land cover: Urban/crop vs Land cover: Savanna		1	25

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
	(SG,R,35)			
Interactions				
Farm is state owned and large vs Farm is private and large	Farm is state owned and large vs Farm is private and large (S,R,54), Farm is state owned and large vs Farm is private and large (S,R,66)		2	764
Semi-extensive management and Water from the river	Semi-extensive management and water from the river (S,R,73)		1	440
Age unknown and Not vaccinated against Toxoplasma	Age unknown and unvaccinated (S,P,71)	1		541
Age of animals and Vaccination status	Age of animals and Vaccination status (S,R,77)		1	541
Other				
	Basic educational level of farmer (elementary school or lower) (SG,P,35), Farm age < 2 years vs Farm age > 10 years (SG,R,50), Location with seropositive animals (SG,R,28), Treatment with albendazoles vs Treatment with salicylanilides (SG,P,35), Toxoplasma-vaccination status unknown (S,R,71)	2	3	25, 541, 993

[a] Species under study: S, sheep; G, goat; SG, sheep and goat at the same time; [b] Direction of effect: R, risk; P, protective; [c] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good; [d] asl: above sea level.

Supplementary Table S4: Results on risk factor analyses chicken

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Breed				
Broiler	[Broilers had a significantly lower seroprevalence than breeder and layer chicken (R,23)]	[1]		479
Extent of specialization				
Backyard farming	[Backyard chicken have a higher risk of being seropositive compared to chicken reared at large farms (R,22)]		[1]	518
Level of confinement				
Outside stable access	[Free range chicken have a higher risk of being seropositive compared to chicken reared at large farms (R,23), Free-range chicken have a higher risk of being seropositive than caged chicken (R,16)]		[2]	479, 683

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S5: Results on risk factor analyses in equids

	Details on variables (Direction of effect[a], Quality score[b]) [Variable from a study of poor quality]	**Number of studies reporting a… [Number of studies of poor quality reporting a…]**		**References**
Categories of reported risk factors		**protective effect**	**risk effect**	
Extent of specialization				
Low level of specialization	Presence of domestic ruminants (R,46)		2	495
Related to geographic localization				
Rural location	[Horses reared in rural areas have a higher risk of being seropositive compared to horses reared in urban areas (R,23)]		[1]	490
Purpose of livestock				
Use of individual animal	Horse used for agricultural work vs Horse used for shows (R,53), [Equid used for farming vs Equid used for racing (R, 22)]		1+[1]	622,1379

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S6: Strength of association between presence of cats and positivity of farms or farm animals. Table is restricted to those studies providing Odds ratio as an outcome value.

Refid	Study type	Study species	Study location	Outcome [Variable from a study of poor quality]	Odds ratio	95% Confidence interval	Statistical Significance
604	Cross-sectional-Multivariable	Pigs	Europe	Cats presence (R,84)	1.61	1.12-2.34	0.01
604	Cross-sectional-Univariable	Pigs	Europe	Cats' presence (R,76)	1.37	1.16–1.60	<0.001
621	Cross-sectional-Univariable	Pigs	Europe	Presence of cats (R,70)	11.3	3.2-19.3	0.01
1011	Cross-sectional-Univariable	Pigs	North America	Cats present (R,64)	2.6	2.0-3.38	<0.001
1380	Cross-sectional-Univariable	Pigs	Europe	There are cats on farm (R,38)	5.15	1.96 - 13.52	<0.001
1380	Cross-sectional-Univariable	Pigs	Europe	At date of sampling cats were on farm (R,38)	4.47	1.82 - 10.95	0.001
1380	Cross-sectional-Univariable	Pigs	Europe	Use of cats to fight rodents (R,38)	2.44	1.17 - 5.08	0.017
738	Cross-sectional-Multivariable	Small ruminants	Europe	Cats on farm (S,R,47)	2.8	1.7–4.5	<0.001
993	Cross-sectional-Multivariable	Small ruminants	Europe	Presence of cats (SG,R,50)	2.31	1.23-4.29	0.009
531	Cross-sectional-Multivariable	Small ruminants	Asia	Presence of cats (SG,R,55)	4.74	1.58 – 14.21	0.01

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S7: Strength of association between number of cats and positivity of farms or farm animals. Table is restricted to those studies providing Odds ratio as an outcome value..

Refid	Study type	Study species	Study location	Outcome [Variable from a study of poor quality]	Reference situation	Odds ratio	95% Confidence interval	Statistical Significance
749	Case-control-Multivariable	Pigs	Europe	More than 3 cats present on farm (R,56)	NA	3.24	NA	0.04
1380	Cross-sectional-Univariable	Pigs	Europe	Actual number of cats present on farm: 1-2 (R,38)	Actual number of cats present on farm: zero	3.2	1.08 - 9.46	0.036
1380	Cross-sectional-Univariable	Pigs	Europe	Actual number of cats present on farm: 3-4 (R,38)	Actual number of cats present on farm: zero	7.29	2.49 -21.34	< 0.001
1380	Cross-sectional-Univariable	Pigs	Europe	Actual number of cats present on farm: 5-15 (R,38)	Actual number of cats present on farm: zero	7.03	2.36 - 20.99	< 0.001
1380	Cross-sectional-Univariable	Pigs	Europe	Previous number of cats present on farm: 1-2 (R,38)	Previous number of cats present on farm: zero	3.68	1.25 - 10.82	0.018
1380	Cross-sectional-Univariable	Pigs	Europe	Previous number of cats present on farm: 3-4 (R,38)	Previous number of cats present on farm: zero	6.08	2.05 - 18.01	0.001
1380	Cross-sectional-Univariable	Pigs	Europe	Previous number of cats present on farm: 5-15 (R,38)	Previous number of cats present on farm: zero	7.27	2.46 - 21.51	< 0.001

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S8: Strength of association between low level of confinement (outside stable access, pastured, lower level of management intensity) and positivity of farms or farm animals

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
479	Cross-sectional-Univariable	Chicken	Asia	Outside stable access	[Free range chicken had a higher seroprevalence compared to caged chickens (R,23)]	NA	NA	NA	<0.01
683	Cross-sectional-Univariable	Chicken	Asia	Outside stable access	[Free-range chicken had a higher risk of being seropositive than caged chicken (R,16)]	NA	NA	NA	<0.001
474	Cross-sectional-Univariable	Pigs	Europe	Pastured	Pigs reared free-range vs Pigs reared intensively (R,30)	Odds ratio	17.6	2.4 - 129.8	<0.05
621	Cross-sectional-Univariable	Pigs	Europe	Outside stable access	Outdoor facilities for sows (R,70)	Odds ratio	9.7	1.7-17.73	0.02
706	Cross-sectional-Univariable	Pigs	Europe	Pastured	Pigs reared free-range vs Pigs reared intensive (R,46)	Odds ratio	15.8	2.0-124	0.009
764	Cross-sectional-Univariable	Pigs	Europe	Outside stable access	Animals have access to outside pens vs Animals in stable only (R,55)	Odds ratio	2.22	1.27–3.90	0.005
1011	Cross-sectional-Univariable	Pigs	North America	Pastured	Sows are entirely pastured vs Sows are kept in total confinement (R,64)	Odds ratio	15.2	11.23-20.62	<0.001
1011	Cross-sectional-Univariable	Pigs	North America	Outside stable access	Sows kept in partial confinement vs Sows kept in total confinement (R,64)	Odds ratio	29.2	22.26-38.45	<0.001
1011	Cross-sectional-Univariable	Pigs	North America	Pastured	Sows are entirely pastured vs Sows kept in partial confinement (P,64)	Odds ratio	0.5	0.39-0.69	<0.001
1011	Cross-sectional-Univariable	Pigs	North America	Pastured	Sows are entirely pastured or kept in partial confinement vs Sows kept in total confinement (R,64)	Odds ratio	23	16.48-35.37	<0.001
1011	Cross-sectional-Univariable	Pigs	North America	Pastured	Sows are entirely pastured or kept in partial confinement vs Sows are entirely pastured or kept in partial confinement (R,64)	Odds ratio	38.9	23.8-64.3	<0.001

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
1011	Cross-sectional-Univariable	Pigs	North America	Pastured	Sows are entirely pastured or kept in partial confinement vs Sows kept in total confinement (R,64)	Odds ratio	2.4	1.2-4.7	NA
194	Cross-sectional-Univariable	Pigs	Europe	Outside stable access	Farms with animal-friendly systems (organic & free-range) have a higher seroprevalence than conventional farms (R,54)	NA	NA	NA	<0.02
721	Cross-sectional-Univariable	Pigs	North America	Outside stable access	[Extensive Antibiotic-free pig production vs conventional intensive production (R,22)]	NA	NA	NA	0.001
578	Cross-sectional-Univariable	Pigs	Asia	Outside stable access	Pigs reared in partially open confinements had a higher risk of being seropositive compared to those from closed confinements (R,44)	NA	NA	NA	≤0.05
578	Cross-sectional-Univariable	Pigs	Asia	Outside stable access	Pigs allowed to scavenge once/day had a higher risk of being positive compared to those allowed to scavenge multiple/per day (R,44)	NA	NA	NA	≤0.01
1380	Cross-sectional-Univariable	Pigs	Europe	Outside stable access	Farms keeping swine in huts (R,38)	Odds ratio	4.9	1.11 - 21.63	0.036
503	Cross-sectional-Univariable	Small ruminants	South America	Lower level of management intensity	Semi-intensive management vs Intensive management (G,R,26)	Relative risk	2.88	1.38 – 6.03	<0.05
636	Cross-sectional-Univariable	Small ruminants	South America	Lower level of management intensity	Extensive production system vs Semi-extensive production system (S,R,42)	Odds ratio	1.68	1.08 - 2.62	0.02017

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
745	Cross-sectional-Univariable	Small ruminants	South America	Lower level of management intensity	Sheep from extensive farms vs Sheep from intensive and semi-intensive farms (S,P,33)	Odds ratio	0.35	0.2208-0.5658	<0.001
1386	Cross-sectional-Univariable	Small ruminants	Europe	Lower level of management intensity	Access to outside pens vs Stable (G,R,45)	Odds ratio	2	1.08-3.71	0.002
1386	Cross-sectional-Univariable	Small ruminants	Europe	Lower level of management intensity	Access to pasture vs Stable (G,R,45)	Odds ratio	2.38	1.43-3.96	0.002
764	Cross-sectional-Multivariable	Cattle	Europe	Outside stable access	Cattle have access to outside pens vs Cattle are in stable only (P,69)	Odds ratio	0.37	0.21–0.67	0.001
764	Cross-sectional-Univariable	Cattle	Europe	Outside stable access	Cattle have access to outside pens vs Cattle are in stable only (P,55)	Odds ratio	0.47	0.29–0.74	<0.001

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S9: Strength of association between high level of management intensity and positivity of small ruminants (farms or farm animals)

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
440	Cross-sectional-Univariable	Small ruminants	Africa	Higher level of management intensity	Agro-pastoral farming system vs Pastoral farming system (S,R,50)	Odds ratio	2.73	1.40 - 5.33	NA
440	Cross-sectional-Univariable	Small ruminants	Africa	Higher level of management intensity	Sedentary farming system vs Pastoral farming system (S,R,50)	Odds ratio	11.12	5.99 - 20.64	<0.001
471	Cross-sectional-Multivariable	Small ruminants	Europe	Higher level of management intensity	Management type intensive vs Management type extensive (SG,R,51)	Odds ratio	3.25	1.07-9.87	0.037408
471	Cross-sectional-Multivariable	Small ruminants	Europe	Higher level of management intensity	Management type semi-intensive vs Management type extensive (SG,P,51)	Odds ratio	0.49	0.33-0.73	0.000477
993	Cross-sectional-Univariable	Small ruminants	Europe	Intensively managed	Intensive management (SG,R,50)	Odds ratio	3.1	1.33-7.17	0.009
697	Cross-sectional-Univariable	Small ruminants	Europe	Higher level of management intensity	[Under intensive management conditions sheep had a higher risk of being seropositive (S,R,20)]	NA	NA	NA	<0.001

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S10: Strength of association between variables suggesting feed contamination is possible and positivity of farms or farm animals

Refid	Study type	Study species	Study location	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
749	Case-control-Multivariable	Pigs	Europe	Roughage not covered (R,56)	Odds ratio	13.45	NA	<0.001
38	Cross-sectional-Univariable	Pigs	North America	Type of feeder manual: higher seroprevalence vs Type of feeder automatic (R,46)	NA	NA	NA	0.003
38	Cross-sectional-Multivariable	Pigs	North America	Manual feeder vs Automatic feeder (P,60)	Odds ratio	0.18	0.04 – 1.32	0.018
1380	Cross-sectional-Univariable	Pigs	Europe	Storage of fodder in open containers (R,38)	Odds ratio	4.36	1.05 - 18.08	0.042
1380	Cross-sectional-Univariable	Pigs	Europe	Silo is open (R,38)	Odds ratio	3.11	1.2 - 8.08	0.02
1380	Cross-sectional-Univariable	Pigs	Europe	Feeding is manually (R,38)	Odds ratio	4.7	2.22 - 9.95	< 0.001
1386	Cross-sectional-Univariable	Small ruminants	Europe	Manger and trough, pasture vs Manger, manger and trough (G,R,45)	Odds ratio	1.85	1.17-2.94	0.009

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good
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Supplementary Table S11: Strength of association between variables suggesting feed contamination is unlikely and positivity of farms or farm animals

Refid	Study type	Study species	Study location	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
1380	Cross-sectional-Univariable	Pigs	Europe	Storage of fodder in closed containers (R,38)	Odds ratio	3.52	1.39 - 8.88	0.008
1380	Cross-sectional-Univariable	Pigs	Europe	Silo is closed (P,38)	Odds ratio	0.39	0.21 -0.75	0.004
1380	Cross-sectional-Univariable	Pigs	Europe	Storage of fodder is generally closed (P,38)	Odds ratio	0.5	0.26 - 0.95	0.039
1380	Cross-sectional-Univariable	Pigs	Europe	Feeding is automated (P,38)	Odds ratio	0.23	0.12 - 0.43	< 0.001

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S12: Strength of association between variables on surface and tap water and positivity of farms or farm animals

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
404	Cross-sectional-Univariable	Pigs	Europe	Surface water	Water supply from shallow wells vs Municipal water supply (R,58)	Odds ratio	2.8	1.03–7.72	0.07
578	Cross-sectional-Univariable	Pigs	Asia	Surface water	Pigs provided surface water had a higher risk of being seropositive compared to pigs provided water from tap or well (R,44)				<=0.05
738	Cross-sectional-Multivariable	Small ruminants	Europe	Surface water	Use of surface water sources vs Use of water from wells (S,R,47)	Odds ratio	1.8	1.1–3.1	0.021
1386	Cross-sectional-Univariable	Small ruminants	Europe	Surface and tap water vs only tap water	Public and/or well/lake/stream vs Public water distribution (G,R,45)	Odds ratio	2.44	1.48-4.04	0.001
440	Cross-sectional-Multivariable	Small ruminants	Africa	Tap water	Tap water vs Water from river, well, lake, pond (S,R,73)	Odds ratio	4.07	1.07 - 15.42	0.039
440	Cross-sectional-Univariable	Small ruminants	Africa	Tap water	Tap water vs Water from river, pond, well, lake ("Mixed") (S,R,50)	Odds ratio	10.28	2.96 - 35.71	<0.001
471	Cross-sectional-Multivariable	Small ruminants	Europe	Tap water	Water from the public supply (SG,R,51)	Odds ratio	3.38	1.23-9.23	0.01781
471	Cross-sectional-Univariable	Small ruminants	Europe	Tap water	Water from the public supply (SG,R,51)	Odds ratio	4.562	1.68-12.4	0.0029

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Supplementary Table S13: Strength of association between variables on rodents and positivity of farms or farm animals

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
404	Cross-sectional-Univariable	Pigs	Europe	No rodent control	No rodenticides used (R,58)	Odds ratio	2.71	1.1–6.64	0.03
404	Cross-sectional-Univariable	Pigs	Europe	No rodent control	No rodenticides used (R,58)	Odds ratio	1.57	1.23–2.02	<0.0001
604	Cross-sectional-Multivariable	Pigs	Europe	No rodent control	Rodent control (R,84)	Odds ratio	1.93	1.04-3.60	0.04
604	Cross-sectional-Univariable	Pigs	Europe	No rodent control	No rodent control (R,76)	Odds ratio	1.54	1.18–2.02	<0.001
1008	Cross-sectional-Multivariable	Pigs	North America	Seropositivity of rodents	Seroprevalence in house mice (R,57)	beta value, regression coefficient	0.342	0.095	0.0023
1008	Cross-sectional-Univariable	Pigs	North America	Seropositivity of rodents	House mouse *T. gondii* seroprevalence (R,56)	NA	NA	NA	<0.001
1008	Cross-sectional-Univariable	Pigs	North America	Seropositivity of rodents	House mouse *T. gondii* seroprevalence (R,56)	NA	NA	NA	0.05
158	Experimental field study	Pigs	Europe	Rodent control	Duration of rodent control (P,37)	beta value, regression coefficient	-0.01	0.0056	0.012
630	Cross-sectional-Multivariable	Pigs	North America	Rodent control	Farm uses traps, bait, poison, exterminator against rodents (P,74)	Estimate, Vuong statistic	0.37	0.21	<0.0001

Refid	Study type	Study species	Study location	Outcome category	Outcome [Variable from a study of poor quality]	Type of outcome value	Outcome value	95% Confidence interval	Statistical Significance
1011	Cross-sectional-Univariable	Pigs	North America	Rodent control	Use of chemicals, traps or destruction of habitats against rodents vs Use of cats or rodent proof containers (R,64)	Odds ratio	2	1.4-2.8	<0.001
38	Cross-sectional-Univariable	Pigs	North America	No presence of rodents	No presence of rodents: higher seroprevalence vs Presence of rodents (P,46)	NA	NA	NA	<0.001
745	Cross-sectional-Univariable	Small ruminants	South America	Presence of rodents	Presence of rodents in the food room (S,R,33)	Odds ratio	6.3	2.4-17.51	0.001
945	Cross-sectional-Multivariable	Small ruminants	Europe	Rodent control	Use of mouse poison (S,R,55)	Odds ratio	2.26	1.02-5.0	0.044

[a] Direction of effect: R, risk; P, protective; [b] Quality score of a study reporting a particular risk or protective effect: ≤ 28, poor; > 28-< 50, average; > 50, good.

Appendix F. All references included in the database by reference identification number (Refid) with status (Included: WP2, WP3, WP2 and WP3; Excluded: Title Screening, Abstract Screening, Initial screening Full-text, WP2 Task Identification, WP3 Eligibility, Additional Exclusion WP2; Quarantine) and bibliographical information

Refid	Status	Bibliography
1	WP2	. Detection of toxoplasma gondii DNA in sheep and goat milk in northwest of Iran by PCR-RFLP. #journal#. #year#. #volume#:#pages#
2	WP3	. Epidemiological study of Toxoplasma gondii infection among cattle in Northern Poland. #journal#. #year#. #volume#:#pages#
3	WP2	. Detection of acute Toxoplasmosis in pigs using loop-mediated isothermal amplification and quantitative PCR. #journal#. #year#. #volume#:#pages#
4	Abstract Screening	D. Anastasia, P. Elias, P. Nikolaos, K. Charilaos, G. Nektarios. Toxoplasma gondii and Neospora caninum seroprevalence in dairy sheep and goats mixed stock farming. Vet Parasitol. 2013. 198:387-90
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6	Initial screening Full-text	. Some risk factors for reproductive failures and contribution of Toxoplasma gondii infection in sheep and goats of Central Ethiopia: A cross-sectional study. #journal#. #year#. #volume#:#pages#
7	Title screening	. Seroprevalence of Toxoplasma Gondii among schizophrenics at Hospital Kajang. #journal#. #year#. #volume#:#pages#
8	Title screening	. Development of Toxoplasma gondii in skeletal muscle cells depends on differentiation of the host cell. #journal#. #year#. #volume#:#pages#
9	Title screening	. Incidence of Toxoplasma gondii infection in the paediatric population of Athens. #journal#. #year#. #volume#:#pages#
10	Title screening	. Toxoplasma gondii increases the expression of S-100 and GFAP in rat colonic myenteric plexus. #journal#. #year#. #volume#:#pages#
11	Title screening	. Insights into the cell cycle regulation of differentiated skeletal muscle cells after infection with toxoplasma gondii. #journal#. #year#. #volume#:#pages#
13	Title screening	. Distribution of lesions and identification of parasites by immunohistochemistry in cases of acute toxoplasmosis in New World primates and prosimians in captivity in Mexico. #journal#. #year#. #volume#:#pages#
14	Title screening	. Genetic and virulence characterisation of Toxoplasma gondii strains isolated from pigeons in Lisbon region. #journal#. #year#. #volume#:#pages#
15	Title screening	. Seropositivity rate of Toxoplasma gondii infection in renal transplant recipients using IFA method. #journal#. #year#. #volume#:#pages#
16	Initial screening Full-text	. Competitive ELISA for Toxoplasma gondii Zoonoses. #journal#. #year#. #volume#:#pages#
17	Title screening	. Toxoplasma gondii seroprevalence in epileptic patients in Iran. #journal#. #year#. #volume#:#pages#
18	Title screening	. A seroprevalence study of toxoplasmosis in pregnant women who referred to rural and urban health care centers. #journal#. #year#. #volume#:#pages#

Refid	Status	Bibliography
19	Title screening	. Manage of toxoplasma seroconversion in pregnant women and perinatal outcomes. #journal#. #year#. #volume#:#pages#
20	Quarantine	Manage of toxoplasma seroconversion in pregnant women and perinatal outcomes
21	Title screening	. Decreased seroprevalence and age-specific risk factors for toxoplasmosis in the Netherlands between 1995-1996 and 2006-2007. #journal#. #year#. #volume#:#pages#
22	Initial screening Full-text	. Risk factors of Toxoplasma infection in Chaharmahal va Bakhtiyari Province, southwest of Iran. #journal#. #year#. #volume#:#pages#
23	Quarantine	Genetic characterization of Toxoplasma gondii from pigs from different localities in China by PCR-RFLP
24	Title screening	C. Alvarado-Esquivel, L. F. Sanchez-Anguiano, C. A. Arnaud-Gil, J. C. Lopez-Longoria, L. F. Molina-Espinoza, S. Estrada-Martinez, O. Liesenfeld, J. Hernandez-Tinoco, A. Sifuentes-Alvarez, C. Salas-Martinez. Toxoplasma gondii infection and suicide attempts: a case-control study in psychiatric outpatients. J Nerv Ment Dis. 2013. 201:948-52
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26	WP2	F. Mancianti, S. Nardoni, C. D'Ascenzi, F. Pedonese, L. Mugnaini, F. Franco, R. Papini. Seroprevalence, detection of DNA in blood and milk, and genotyping of Toxoplasma gondii in a goat population in Italy. Biomed Res Int. 2013. 2013:905326
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32	WP2	K. Chumpolbanchorn, A. J. Lymbery, L. J. Pallant, S. Pan, Y. Sukthana, R. C. Thompson. A high prevalence of Toxoplasma in Australian chickens. Vet Parasitol. 2013. 196:209-11
33	Abstract Screening	. Toxoplasma gondii infection in free-range chicken: Mini-review and seroprevalence study in Oyo state, Nigeria. #journal#. #year#. #volume#:#pages#

Refid	Status	Bibliography
34	Quarantine	Prevalence and genetic characterization of Toxoplasma gondii in house sparrows (Passer domesticus) in Lanzhou, China
35	Title screening	S. H. Hong, Y. I. Jeong, J. Y. Kim, S. H. Cho, W. J. Lee, S. E. Lee. Prevalence of Toxoplasma gondii infection in household cats in Korea and risk factors. Korean J Parasitol. 2013. 51:357-61
36	Quarantine	SAG2A protein from Toxoplasma gondii interacts with both innate and adaptive immune compartments of infected hosts
37	Quarantine	Seroprevalence and risk factors of toxoplasmosis in cattle from extensive and semi-intensive rearing systems at Zona da Mata, Minas Gerais state, Southern Brazil
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39	Quarantine	Seroprevalence of Toxoplasma gondii in horses and donkeys in Yunnan Province, Southwestern China
40	Quarantine	Detection of toxoplasma gondii in raw caprine, ovine, buffalo, bovine, and camel milk using cell cultivation, cat bioassay, capture ELISA, and PCR methods in Iran
41	Title screening	J. P. Dubey, S. Choudhary, O. C. Kwok, L. R. Ferreira, S. Oliveira, S. K. Verma, D. R. Marks, K. Pedersen, R. M. Mickley, A. R. Randall, D. Arsnoe, C. Su. Isolation and genetic characterization of Toxoplasma gondii from mute swan (Cygnus olor) from the USA. Vet Parasitol. 2013. 195:42-6
42	WP2	W. D. Lopes, J. D. Rodriguez, F. A. Souza, T. R. dos Santos, R. S. dos Santos, W. M. Rosanese, W. R. Lopes, C. A. Sakamoto, A. J. da Costa. Sexual transmission of Toxoplasma gondii in sheep. Vet Parasitol. 2013. 195:47-56
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52	Quarantine	Genotyping of Toxoplasma gondii isolates from wild boars in Peninsular Malaysia
53	Abstract Screening	. High Diagnostic efficiency of affinity isolated fraction in camel and cattle toxoplasmosis. #journal#. #year#. #volume#:#pages#
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55	Title screening	E. Z. Gebremedhin, A. H. Abebe, T. S. Tessema, K. D. Tullu, G. Medhin, M. Vitale, V. Di Marco, E. Cox, P. Dorny. Seroepidemiology of Toxoplasma gondii infection in women of child-bearing age in central Ethiopia. BMC Infect Dis. 2013. 13:101
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1692	Title screening	. 8th Annual Congress of the European-Society-of-Veterinary-Clinical-Pathology (ESVCP), Cambridge, UK, September 05 -08, 2006. #journal#. #year#. #volume#:#pages#
1693	Title screening	. Is Toxoplasma gondii a potential risk for traffic accidents in Turkey?*. #journal#. #year#. #volume#:#pages#
1694	Title screening	. In-situ hybridization for the detection and identification of Histomonas meleagridis in tissues. #journal#. #year#. #volume#:#pages#
1695	Title screening	. Primary structure of mature SAG1 gene of an Indonesian Toxoplasma gondii and comparison with other strains. #journal#. #year#. #volume#:#pages#
1696	Quarantine	Cats and goat whey associated with Toxoplasma gondii infection in pigs
1697	Title screening	. Serodiagnosis of trichinellosis: In-house versus commercial ELISA. #journal#. #year#. #volume#:#pages#
1698	Title screening	. Cellular and immunological basis of the host-parasite relationship during infection with Neospora caninum. #journal#. #year#. #volume#:#pages#
1699	Quarantine	Characterization of Toxoplasma gondii from domestic animals from Minas Gerais, Brazil
1700	Abstract Screening	. Toxoplasma gondii and Chlamydophila abortus in caprine abortions in tobago: a sero-epidemiological study. #journal#. #year#. #volume#:#pages#
1701	Title screening	. A genetically diverse but distinct North American population of Sarcocystis neurona includes an overrepresented clone described by 12 microsatellite alleles. #journal#. #year#. #volume#:#pages#
1702	Additional Exclusion WP2	. Toxoplasmosis: The possibility of vertical transmission. #journal#. #year#. #volume#:#pages#
1703	Title screening	. Veterinary and medical aspects of abortion in Danish sheep. #journal#. #year#. #volume#:#pages#
1704	Abstract Screening	. Investigation of Neospora sp and Toxoplasma gondii antibodies in mares and in precolostral foals from Parana State, Southern Brazil. #journal#. #year#. #volume#:#pages#

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1705	Title screening	. Protecting babies: vaccine strategies to prevent foetopathy in Neospora caninum-infected cattle. #journal#. #year#. #volume#:#pages#
1706	Quarantine	Cross-sectional survey on Toxoplasma gondii infection in cattle, sheep and pigs in Serbia: Seroprevalence and risk factors
1707	Title screening	. Is the immune response to Neospora caninum incompatible with pregnancy in cattle?. #journal#. #year#. #volume#:#pages#
1708	Title screening	. Anti-Toxoplasma gondii antibodies in hemodialysis patients receiving long-term hemodialysis therapy in Turkey. #journal#. #year#. #volume#:#pages#
1709	Title screening	. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. #journal#. #year#. #volume#:#pages#
1710	Abstract Screening	. Seroprevalence of T. gondii in sheep from Marrakech, Morocco. #journal#. #year#. #volume#:#pages#
1711	Abstract Screening	. Preliminary characterisation of Toxoplasma gondii isolates from Zimbabwe, with stage-specific monoclonal antibodies. #journal#. #year#. #volume#:#pages#
1712	Initial screening Full-text	. Serological prevalence of Toxoplasma gondii in free-range chickens from Costa Rica. #journal#. #year#. #volume#:#pages#
1713	WP2	. Zoonotic toxoplasmosis in chicken. #journal#. #year#. #volume#:#pages#
1714	Title screening	. Vaccines as a solution for veterinary parasites instead of drugs. #journal#. #year#. #volume#:#pages#
1715	Initial screening Full-text	. Serologic prevalence of Toxoplasma gondii in chickens in Afyon, Turkey. #journal#. #year#. #volume#:#pages#
1716	Quarantine	Toxoplasma gondii: taux de portage chez les ovins de la region de Marrakech (Mnabha) Toxoplasma gondii: level of carriage in sheep of Marrakech region (Mnabha)
1717	Initial screening Full-text	. Seroepidemiologische studien zur toxoplasmose aus human- and veterinarmedizinischer sicht - eine retrospektive der ietzten 25 Jahre in Osterreich Seroepidemiology of Toxoplasma infections in Austria - a retrospective view of human and veterinary medicine over the past 25 years. #journal#. #year#. #volume#:#pages#
1718	Initial screening Full-text	. Intranasal immunisation with Toxoplasma gondii tachyzoite antigen encapsulated into PLG microspheres induces humoral and cell-mediated immunity in sheep. #journal#. #year#. #volume#:#pages#
1719	Initial screening Full-text	. Les avortements chez les petits ruminants Abortion in small ruminants. #journal#. #year#. #volume#:#pages#
1720	Title screening	. Expression of two glycolytic pathway enzymes of the protozoan Eimeria tenella: Enolase and pyruvate kinase. #journal#. #year#. #volume#:#pages#
1721	Title screening	. Immune response and antigen recognition in non-pregnant ewes experimentally infected with Neospora caninum tachyzoites. #journal#. #year#. #volume#:#pages#
1722	Title screening	. Fetal death: comparative aspects in large domestic animals. #journal#. #year#. #volume#:#pages#
1723	Title	. Toxoplasmosis in pregnant women in Sanliurfa, southeastern Anatolia City,

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	screening	Turkey. #journal#. #year#. #volume#:#pages#
1724	Abstract Screening	. Seroprevalence of toxoplasmosis in sheep in Aydin region in Turkey. #journal#. #year#. #volume#:#pages#
1725	Title screening	. Conservation of Babesia bovis small heat shock protein (Hsp20) among strains and definition of T helper cell epitopes recognized by cattle with diverse major histocompatibility complex class II haplotypes. #journal#. #year#. #volume#:#pages#
1726	Title screening	. Serodiagnosis of Neospora caninum infection in cattle by enzyme-linked immunosorbent assay with recombinant truncated NcSAG1. #journal#. #year#. #volume#:#pages#
1727	Abstract Screening	. From cells to signaling cascades: Manipulation of innate immunity by Toxoplasma gondii. #journal#. #year#. #volume#:#pages#
1728	Abstract Screening	. Prevalence of antibodies to Toxoplasma gondii in four breeds of cattle at Ibadan, Nigeria. #journal#. #year#. #volume#:#pages#
1729	Title screening	. Prevalence of toxoplasmosis in Van cats in Turkey. #journal#. #year#. #volume#:#pages#
1730	Abstract Screening	. Immunodiagnosis of primary Toxoplasma gondii infection in sheep by the use of a P30 IgG avidity ELISA. #journal#. #year#. #volume#:#pages#
1731	Title screening	. Correlation between Ornithine Decarboxylase Activity and Toxoplasma Gondii Proliferation in Cultured Embrionic Retina. #journal#. #year#. #volume#:#pages#
1732	WP2	. Isolation of Toxoplasma gondii from goats with a history of reproductive disorders and the prevalence of Toxoplasma and chlamydial antibodies. #journal#. #year#. #volume#:#pages#
1733	Additional Exclusion WP2	. Detection of Chlamydophila (Chlamydia) abortus and Toxoplasma gondii in smears from cases of ovine and caprine abortion by the streptavidin-bio tin method. #journal#. #year#. #volume#:#pages#
1734	Additional Exclusion WP2	. Aborte beim kleinen Wiederkaeuer in der Schweiz: Untersuchungen waehrend zwei Ablammperioden (1996-1998) unter besonderer Beachtung des Chlamydienabortes. Abortion in small ruminants in Switzerland: Investigations during two lambing seasons with special regard to Chlamydiae. #journal#. #year#. #volume#:#pages#
1735	Title screening	. Serological investigation of aborted sheep and pigs for infection by Neospora caninum. #journal#. #year#. #volume#:#pages#
1736	Title screening	. Neospora caninum: A cause of immune-mediated failure of pregnancy?. #journal#. #year#. #volume#:#pages#
1737	Abstract Screening	. Seroprevalence of Neospora, Toxoplasma gondii and Sarcocystis neurona antibodies in horses from Jeju island, South Korea. #journal#. #year#. #volume#:#pages#
1738	Title screening	. Sensitive and specific identification of Neospora caninum infection of cattle based on detection of serum antibodies to recombinant Ncp29. #journal#. #year#. #volume#:#pages#
1739	Quarantine	Single tube nested PCR for the detection of Toxoplasma gondii in fetal tissues from naturally aborted ewes
1740	Abstract Screening	. Analysis of IgG response to experimental infection with RH Toxoplasma gondii in goats. #journal#. #year#. #volume#:#pages#
1741	Title	. Identification of circulating antigens, including an immunoglobulin binding

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	screening	protein, from Toxoplasma gondii tissue cyst and tachyzoites in murine toxoplasmosis. #journal#. #year#. #volume#:#pages#
1742	Initial screening Full-text	. Toxoplasma gondii IgG antibodies in chickens and pigs of intensive breed. #journal#. #year#. #volume#:#pages#
1743	Initial screening Full-text	. Seroprevalence of Toxoplasma gondii in domestic livestock from a small farm of Santa Catarina State, Brazil. #journal#. #year#. #volume#:#pages#
1744	Initial screening Full-text	. IgM response in goats experimentally infected with RH strain of Toxoplasma gondii. #journal#. #year#. #volume#:#pages#
1745	Abstract Screening	. Prevalence of Toxoplasma gondii antibodies in sera of turkeys, chickens, and ducks from Egypt. #journal#. #year#. #volume#:#pages#
1746	Title screening	. The antigenic composition of Neospora caninum. #journal#. #year#. #volume#:#pages#
1747	Title screening	. Neosporosis in Switzerland. #journal#. #year#. #volume#:#pages#
1748	Initial screening Full-text	. Toxoplasmosis and iodine deficiency in Angora goats. #journal#. #year#. #volume#:#pages#
1749	Title screening	. Immunogenicity of recombinant BCG producing the GRA1 antigen from Toxoplasma gondii. #journal#. #year#. #volume#:#pages#
1750	Title screening	. Cellular immune responses in cattle experimentally infected with Neospora caninum. #journal#. #year#. #volume#:#pages#
1751	Abstract Screening	. A chicken anti-conoid monoclonal antibody identifies a common epitope which is present on motile stages of Eimera, Neospora, and Toxoplasma. #journal#. #year#. #volume#:#pages#
1752	Quarantine	The incidence and economic significance of ovine toxoplasmosis in Uruguay
1753	Title screening	. The consequences of the intracellular retention of pathogen-derived T-cell-independent antigens on protein presentation to T cells. #journal#. #year#. #volume#:#pages#
1754	Initial screening Full-text	. Toxoplasma gondii infection in sheep and cattle. #journal#. #year#. #volume#:#pages#
1755	Initial screening Full-text	. A survey of Toxoplasma gondii antibodies in goats and cattle in Lampung Province, Indonesia. #journal#. #year#. #volume#:#pages#
1756	Abstract Screening	. Comparison of indirect immunofluorescent antibody test and modified direct agglutination test methods for detection of Toxoplasma gondii antibodies in adult sheep in Spain. #journal#. #year#. #volume#:#pages#
1757	Abstract Screening	. Effect of Toxoplasma gondii infection on the development of pregnancy and on endocrine foetal-placental function in the goat. #journal#. #year#. #volume#:#pages#
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1760	Title screening	. Rapid and sensitive identification of Neospora caninum by in vitro amplification of the internal transcribed spacer 1. #journal#. #year#. #volume#:#pages#
1761	Title screening	. Chemotherapy of human and animal coccidioses: State and perspectives. #journal#. #year#. #volume#:#pages#
1762	Title screening	. Serologic responses of cattle and other animals infected with Neospora caninum. #journal#. #year#. #volume#:#pages#
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1766	Title screening	. Neosporosis. #journal#. #year#. #volume#:#pages#
1767	Title screening	. Encephalomyelitis due to a Sarcocystis neurona-like protozoan in a rhesus monkey (Macaca mulatta) infected with simian immunodeficiency virus. #journal#. #year#. #volume#:#pages#
1768	Abstract Screening	. Immunization against Toxoplasma gondii. #journal#. #year#. #volume#:#pages#

ABBREVIATIONS

ANSES –USC EpiToxo	French Agency for Food, Environmental and Occupational health and Safety
BA	Mouse Bioassay
BP	Base pairs
Cq	Cycle for quantification in qPCR
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
IgG	Immunoglobulines isotype G
IgM	Immunoglobulines isotype M
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
qPCR	Quantitative Polymerase Chain Reaction
RE	Repetitive Element
RIVM	National Institute for Public Health and the Environment
RVC	Royal Veterinary College
SFDT	Sabin-Feldman Dye test
TgSAG1	*Toxoplasma gondii* Surface antigen 1
UASVM CN	University of Agricultural Science and Veterinary Medicine, Cluj-Napoca
WP	Workpackage