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Université Pierre et Marie Curie University of Belgrade

“Complexité du vivant”

Animal health laboratory – ANSES

“Biology”, module “Immuno - biology”

Institute for Medical Research, National Reference Laboratory for Toxoplasmosis

Prevalence and risk factors for toxoplasmosis in pigs and characterization of natural evolution of infection in a pig model

Par: Vitomir Djokic

Thèse de doctorat de : Parasitologie

Dirigée par:

Pascal Boireau – UMR BIPAR, Animal health laboratory, ANSES

Olgica Djurkovic Djakovic – NRL Toxo, Institute for Medical Research

Thesis Supervisor:

Radu Blaga – UMR BIPAR, Ecole Nationale Vétérinaire d’Alfort

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Devant un jury composé de :

1. Christophe Hennequin (Professeur) – Président du jury
2. Marie-Laure Dardé (Professeure) - Rapporteur
3. Walter U. Basso (Professeur) - Rapporteur
4. Joke van der Giessen (DVM PhD Dipl. EVPC) - Examineur
5. Jean Dupouy-Camet (Professeur) - Examineur
6. Biljana Bozic (Professeure) - Examineur
7. Olgica Djurkovic Djakovic (MD/PhD) – Co-directrice de la thèse
8. Pascal Boireau (DVM PhD Dipl. EVPC) – Co-directeur de la thèse

To my parents and my dear sister:

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Résumé

Резиме:

Увод

Паразит из филума *Apicomplexa*, *Toxoplasma gondii* је прилагођен за инфекцију свих топлокрвних животиња, укључујући и човека. Постнатална инфекција је последица ингестије: 1) ткивних цисти које се налазе у мишићима инфицираних животиња, или 2) ооцисти - форми отпорних на услове животне средине, које се могу наћи у води или на контаминираном воћу и поврћу. Болест је углавном бенигног карактера, али може оставити озбиљне последице на плод у случају инфекције током трудноће, или на индивидуе ослабљеног имунског система.

Поред овчетине и пилетине, *T. gondii* паразити се најчешће могу наћи у свињском месу. У условима интензивног това свиња, без обзира на различите зоо-хигијенске нивое обезбеђења фарми, интензитет *T. gondii* инфекције је нижи него на отвореним фармама или у органској производњи у свим европским земљама. Лоша дератизација и присуство мачака на фармама интензивне производње свиња су главни извори *T. gondii* инфекције. Слама као простирка може садржати ооцисте или чак и угинуле глодаре, или у случају да су силоси и дистрибутери отворени, храна за свиње може бити контаминирана. Супротно условима интензивне производње, на отвореним и органским фармама, свиње су изложене *T. gondii* инфекцији кроз контаминирану животну средину, земљиште, храну и воду.

За сада рутинска контрола *T. gondii* инфекције код свиња не постоји нигде у свету, како на нивоу кланице, тако ни на засебним фармама. Стога извори инфекције на фармама још увек не могу са сигурношћу бити проучени. Шта више, не може се са сигурношћу утврдити ни колико је месо инфицираних животиња заступљено на светском тржишту и у људској исхрани. Циљ ове студије је био да опише преваленцу *T. gondii* у свиња одгајаним за људску исхрану у Француској. Изолација паразита из интензивно товлених свиња је показала да је неопходно засебно истражити факторе који утичу на инфекцију животиња у условима стриктно затвореног това. Како би се смањио ризик инфекције људи конзумирањем свињетине анализирана је корелација између имунског одговора свиња и присуства инфективних паразита или паразитске ДНК у јестивим ткивима природно инфицираних животиња. Најзад, осмишљен је ефикасан метод за производњу антигена неопходног за тест модификоване аглутинације (МАТ), како би се омогућила шира употреба репрезентативног теста за животиње.

Методи

За анализу *T. gondii* преваленце код свиња у Француској скупљање узорака је било дизајнирано на основу годишње производње свиња из интензивног и екстензивног това. Укупно је прикупљено 1549 узорака ткивне течности из срца свиња како би се одредила преваленца употребом МАТ теста. Од сакупљених узорака 160 срца је анализирано у биолошком огледу у сврху изолације живих паразита. Добијени резултати су упоређени са сличним студијама које су испитивале *T. gondii* преваленцу код оваца и говеда.

Како би се анализирали фактори ризика за *T. gondii* инфекцију, серуми свиња из интензивног това као и епидемиолошки подаци о узгоју и зоо-хигијенским условима фарми су скупљени за 3595 прасади на сиси, залучених прасади и свиња у тову из западне Француске. Карактеристике узгоја, менаџмента фарми, као и зоотехнички и зоохигијенски подаци су сврстани у три групе фактора ризика. МАТ је коришћен за детекцију специфичних антитела у серумима свиња, за четири двострука разблажења, почевши од 1/6.

За израчунавање подударности између МАТ резултата добијених анализом серума и срчаних сокова, као и однос између резултата серологије и директне изолације паразита или паразитске ДНК (биолошким огледом у мишевима, квантитативним ПЦР-ом на дигестованим срцима или Магнетним Скенирањем ПЦР-ом (МС-ПЦР) дијафрагми свиња) коришћено је 140 узорак из националне (горе описане) студије. Изабрано је 88 МАТ позитивних животиња ($\text{МАТ} \geq 1/6$) и насумично одабраних 52 свиња које су биле серонегативне ($\text{МАТ} \leq 1/6$). Поређење је било могуће између биолошког огледа, квантитативног ПЦР-а на узорцима дигеста срца и МЦ-ПЦР-а на узорцима дијафрагми. Ови резултати су такође поређени са МАТ анализама серума и срчаних сокова како би се одредио однос између концентрације антитела и директног присуства паразита у јестивим ткивима свиња.

У циљу продукције антигена за МАТ анализирано се одржавање и пречишћавање Rh соја *T. gondii* у хуманим фибробластима обреска (human foreskin fibroblasts, HFF), хуманим колон-илеоцекалним ћелијама аденокарцинома (human colon-ileocecal colorectal adenocarcinoma, HCT-8) и хуманим епителијалним колоректалним ћелијама аденокарцинома (human epithelial colorectal adenocarcinoma cells, CaCo-2). Пречишћавање и припрема антигена је рађена по модификованом протоколу Дезмонда и Ремингтона, за умножавање *Toxo* Rh код мишева. За разлику од оригиналног протокола, за пропагацију кроз ћелијску културу, била је потребна само једна генерација паразита, што штеди време и смањује употребу лабораторијских животиња. Такође, за разлику од ослобађања паразита из перитонеума мишева, за описани метод није потребна трипсинизација ћелија да би се добили слободни паразити. Антиген из ћелијске културе је поређен са антигеном из миша коришћењем серума експериментално инфицираних оваца и мишева, као и кардијачног сока природно инфицираних говеда и серума свиња, коза, паса и мачака.

Резултати

Прерачуната серопреваленца *T. gondii* код свиња са интензивних фарми је била 3.0%; и 6.3% код оних гајених у екстензивним условима. Највиша стопа инфекције је била код крмача (13.4%); 2.9% код товленика, а најнижа, 2.6% код прасади. Укупно је изолован 41 сој и уз помоћ методе полиморфизма дужине граничних фрагмената (Restriction Fragment Length Polymorphism, RFLP) утврђено је да сви припадају сојевима типа II. Ризик од инфекције је био преко три пута већи код екстензивно гајених свиња, док је код крмача ризик био скоро пет пута

већи него код товљеника. Од 3595 узорак прикупљених са 60 интензивних фарми у западној Француској, укупна преваленца на индивидуалном нивоу је била 6.9%. Преваленца фарми је била 100% са минимум једном позитивном свињом, а просечан интензитет инфекције је био 6.95%. *T. gondii* преваленца у прасилиштима је била 48%, 49% у свињцима за залучивање прасића и 56.3% у оним за товљење. Мешовити линеарни модел је показао да су прасићи након залучења и они у тову најугроженији и вероватноћа инфекције је 1.61 и 1.74 пута већа у ових категорија него код свиња за клање (p-вредност= 0,0399 и 0,0005). Мале фарме су 2.99 пута угроженије од *T. gondii* него средње и велике фарме. Зграде са инсталираним данским системом уласка дају бољу заштиту од *T. gondii* (OR= 0-44, p-вредност= 0,0018).

Од 144 узорак свиња коришћених за утврђивање корелације између серологије, биолошког огледа и детекције *T. gondii* ПЦР-ом, антитела су детектована код 61% свињских серума и 43% кардијачних сокова. Анализа односа између МАТ резултата и биолошког огледа (receiver operating characteristics analysis, ROC) је подесила најбољи пресек за МАТ разблажење на 1/9, код серума и кардијачних сокова. Слагање (капа вредности) између резултата индиректне дијагностике је било слабо (0.35 95% ИП: 0.18 – 0.52) и умерено (0.54 95% ИП: 0.39 – 0.71) када су, као пресеци, коришћена разблажења 1/6 и 1/12. Када је поређен МАТ на две матрице са директним дијагностичким методама (биолошки оглед на мишевима, квантитавини ПЦР и МС-ПЦР), највећа капа вредност је уочена између МАТ-а ($\geq 1/12$) за кардијачни сок и мишијег биоесеја (0.74). Анализирајући методе директне дијагностике, квантитавини ПЦР на дигесту срца се показао као најсензитивнији са 69 (43.1%) позитивних резултата, затим биолошки оглед са 41 (27.7%) и МС-ПЦР дијафрагме са 27 (16.9%). МС-ПЦР и квантитативни ПЦР су у позитивној корелацији са резултатима мишијег биоесеја. Најбоље слагање нађено је између квантитативног ПЦР-а и биолошког огледа у мишу (0.50), међутим изолација *T. gondii* из серонегативних свиња указује на постојање још увек значајног ризика по потрошаче.

Најбољи антиген за МАТ добијен је када је *T. gondii* умножен у HFF ћелијама. Количина паразита по фласку је била довољна за производњу 1-2ml антигена ($2 - 4 \times 10^8$ фиксираних тахизоита). Капа вредност је показала одлично слагање између антигена произведеног у мишу и оног у HFF ћелијама, када је поређење рађено на серумима експериментално и природно инфицираних животиња (капа вредност између 0.64 - 1), и говеђим кардијачним соковима (капа: 0.81 95% ИП: 0.65 - 0.99). McNamara тест је показао довољне сличности између антигена тестираних на свим узорцима (p-вредност између 0.074 - НА). Солидна корелација (0.78, 95% ИП: 0.71 - 0.84) уочена је када су антигени поређени на свињским серумима, док су остали узорци показали савршену корелацију на свих 8 разблажења (Табела 4).

Закључци

Описана преваленца *T. gondii* доказује да је чак и у контролисаним условима узгоја свиња инфекција могућа и реална. Ова студија показује највећи ризик од инфекције *T. gondii* у периоду после залучења прасади. Мале фарме су подложније паразитској инфекцији. Такође, показано је да дански систем улаза на зградама фарми обезбеђује ефикасну заштиту од *T. gondii*. МАТ може да се користи за тријажу инфицираних и неинфицираних свиња како би се смањио ризик по потрошаче свињетине. Високе негативне предиктивне вредности гарантују идентификацију стварно негативних свиња. Насупрот томе, ниске позитивне предиктивне вредности не оправдавају коришћење МАТ-а за анализу серума свиња, с обзиром на идентификацију великог броја позитивних животиња из чијих узорак паразити нису изоловани. Производња HFF ћелија и пасажа Rh тахизоита не захтева високо обучено особље, нити специјалне просторије, и може да се изведе у лабораторијама са стандардном опремом за ћелијску културу. Коришћење Rh тахизоита из HFF ћелијске културе за МАТ антиген може бити употребљен за епидемиолошке студије *T. gondii* у раличитим животињским врстама и у кланицама тријажу животиња које су носиоци *T. gondii* инфекције.

Abstract:

Background

The parasite from phylum *Apicomplexa*, *Toxoplasma gondii* is adapted for infection of all warm-blooded animals including humans. Infection postnatally occurs through ingestion of i) tissue cysts present in muscles of infected animals, or ii) environmentally resistant forms - oocysts via contaminated water, fruits and vegetables. Disease is generally benign, but infection may have serious consequences for the developing foetus in the case of maternal infection in pregnancy and in immunosuppressed individuals.

Along with sheep and poultry, *T. gondii* parasites are mostly isolated from pig meat. In intensive systems with various levels of confinement, intensity of *T. gondii* infection is still considerably lower than in outdoor or organic farms throughout Europe. Poor rodent control and presence of cats on intensive farms are major sources of *T. gondii* infection for pig. When straw is used for bedding, it can contain oocysts or even rodent carcasses, and if the food silos or distributors are opened, pig nutrition can be contaminated. In contrast to intensive farming, on outdoor and organic farms pigs are exposed to environmental *T. gondii* contamination through soil, food, and water sources.

So far, routine surveillance of pigs for *T. gondii* infection has not been established anywhere in the World, neither in the slaughterhouse nor on the farm-levels. Thus the sources of pig infection on the farms are still unknown. Moreover, it is unknown how big the role of the meat from *T. gondii* infected pigs is in human nutrition.

The aim of this study was to describe the prevalence of *T. gondii* in pigs destined for human consumption in France. The parasite isolation from intensively bred pigs, showed the need to examine separately, risk factors that influence pig contamination in strictly confined conditions. In order to prevent human *T. gondii* infection by consumption of pork, correlation between pig's immune response and presence of parasites or parasitic DNA in edible tissues of naturally infected animals was analysed. Finally, effective method of antigen production for Modified agglutination test (MAT) was devised in order to facilitate access to gold standard test for diagnostic of *T. gondii* infected animals.

Methods

For *T. gondii* prevalence analysis in pigs from France, a survey was designed based on annual numbers of slaughtered pigs from intensive and outdoor farms in France. In total 1,549 samples of cardiac fluids were collected from pig hearts to determine seroprevalence using a MAT. Of those, 160 hearts were bio-assayed in mice to isolate live parasites. Obtained prevalence were compared to results of previous nation-wide *T. gondii* studies on sheep and cattle samples.

To analyse risk factors for *T. gondii* infection of pigs from confined farrow-to-finish farms sera along with rearing and biosecurity practices data were collected from 3595 suckling, weaned piglets and fattening pigs from western France. Breeding features, farm management, as well as technology and biosecurity were analysed as three risk factors classes. MAT was used for detection of

anti *T. gondii* antibodies in pig sera, starting from 1/6 dilution and four two-fold dilutions were further made.

The concordance between MAT on sera and cardiac juices, along with direct detection of parasites or parasitic DNA (mouse bioassay, quantitative PCR (qPCR) on heart digest and Magnetic Capture PCR (MCPCR) on diaphragm) was assessed using 140 nation-wide study samples, from 88 positive animals (MAT titre $\geq 1:6$) and 52 randomly selected seronegatives (MAT $< 1:6$). Comparison was made between mouse bioassay, qPCR on heart digest, and MC-PCR on diaphragm. These results were matched with MAT analyses in order to calculate the relation between direct and indirect detection methods.

For production of MAT antigen, growth and purification of *T. gondii* Rh strain was tried in human foreskin fibroblasts (HFF), human colon-ileocecal colorectal adenocarcinoma (HCT-8) and human epithelial colorectal adenocarcinoma cells (CaCo-2). Purification and preparation of antigen was done by modification of protocol from Desmonts and Remington, for Toxo Rh amplification in mice. In difference to described protocol, for parasites propagation in cell cultures, only one parasitic generation was needed, thus saving the time and use of laboratory animals. Contrary to collection of parasites from mice, no trypsinisation of cells was needed in order to liberate the parasites. Cell culture antigens were compared to mouse derived one on sera from experimentally infected sheep and mice, as well as on cardiac juice from naturally infected cattle and sera from pigs, goats, dogs and cats.

Results

The adjusted seroprevalence in pigs from intensive farms was 3.0%; and 6.3% in outdoor animals. The highest infection rate was in sows (13.4%); 2.9% in fattening pigs, and lowest, 2.6% in piglets. In total 41 strains was isolated and according to Restriction Fragment Length Polymorphism (RFLP) all belong to type II. Risk of infection was more than three times higher for outdoor pigs, while sows had almost five times higher risk than fattening animals.

Out of 3595 samples collected from 60 farrow-to-finish farms in western France, the overall prevalence on individual level was 6.9%. Farm prevalence was 100% with at least one positive pig, and mean intensity of infection on farms was 6.9%. *T. gondii* prevalence in farrowing buildings was 48%, 49% in post-weaning and 56.3% in fattening pens. Mixed linear model showed that piglets after weaning and in fattening are the most endangered and that the chances of acquiring infection in this period is 1.61 and 1.74 times higher than in finishing pigs (p -value= 0.0399 and 0.0005, respectively). Small farms are 2.99 times more endangered by *T. gondii* than middle sized and big producers. Pens with installed Danish entry systems give higher protection against *T. gondii* (OR=0.44, p -value=0.0018).

Out of the 140 pig samples used for concordance between serology, bioassay and PCR-based detection of *T. gondii*, antibodies were detected in 61% of pig sera and in 43% of cardiac juices. Receiver operating characteristics analysis (ROC) set the best cut off, both for sera and cardiac juices, at 1/9 MAT dilution. The concordance (kappa values) between indirect diagnostic results was fair

(0.35 95%CI: 0.18 – 0.52) and moderate (0.54 95%CI: 0.39 – 0.71) when 1/6 and 1/12 dilutions were used as cut offs, respectively. When compared the MAT on two matrices with the direct detection methods (mouse bioassay, qPCR and MC-PCR), the highest kappa value was observed between MAT ($\geq 1/12$) on cardiac juice and mouse bioassay (0.74). Analysing the direct detection methods, qPCR on heart digest was the most sensitive with 69 (43.1%) positive results, followed by mouse bioassay with 41 (27.7%) and MC-PCR on diaphragm with 27 (16.9%). Both qPCR and MC-PCR are in positive concordance with the findings of mouse bioassay. The best concordance was found between qPCR and mouse bioassay (0.50), however the isolation of *T. gondii* from a seronegative pig indicate still high risk for consumers.

The best antigen for MAT was obtained when Toxo Rh tachyzoites were amplified in HFF cells. Per flask quantity of parasites was sufficient for production of 1-2ml of antigen ($2-4 \times 10^8$ fixed tachyzoites). The kappa value showed excellent concordance between antigens produced in mice and HFF cultures, both on sera from experimentally and naturally infected animals (kappa value between 0.64 - 1), and bovine cardiac fluids (kappa: 0.81 95%CI: 0.65-0.99). McNamara test demonstrated sufficient similarities between antigens tested on all samples (p-value between 0.074-NA). The substantial correlation (0.78, 95%CI: 0.71-0.84) was observed when antigens were compared on pig sera, while all other samples showed perfect correlations throughout all 8 dilutions (Table 4).

Conclusions

Described *T. gondii* prevalence provides further evidence that even in confined conditions of pig breeding, infection occurs. Current study shows that the highest risk of acquiring *T. gondii* is at the end of weaning period. Small productions demonstrated higher susceptibility to parasitic infection. This study also shows that Danish entries on farm buildings provide effective protection against *T. gondii*. MAT can be used to indicate the risk for humans consuming pork. High negative prediction value guaranties identification of truly negative pig. Contrary, low positive predictive value disagrees with use of MAT on sera considering economic justification. Production of HFF cells and passage of Rh tachyzoites does not require highly trained staff or special facilities, and can be performed in laboratories equipped with standard cell culture devices. Antigen produced from Rh tachyzoites amplified in HFF cells can be used for animal epidemiological studies and *T. gondii* animal screening in slaughterhouses

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Introduction

Toxoplasma gondii is one of the world's most widespread organisms. The parasite from phylum *Apicomplexa* is adapted for infection of all warm-blooded animals including humans. Infection occurs through the ingestion of tissue cysts present in muscles of infected animals, or by environmentally resistant forms, oocysts via contaminated water, fruits and vegetables. Due to an observed decrease of *T. gondii* prevalence in humans, all over the World, risk of acquiring the parasite's infection in immune deficiency conditions rises (1). New culinary trends, in which raw or undercooked meat is used, nowadays represent one of the major risk factors for human infection (2, 3). Disease is generally benign, but infection may have serious consequences for the developing foetus in the case of maternal infection in pregnancy and in immunosuppressed individuals (4). In those individuals of undeveloped or compromised immune system, the parasite can cause disease *Toxoplasmosis*. Fever, lymphadenitis, and life-long infection of nervous system and eye or even life-threatening conditions are characteristics of *Toxoplasmosis*. Due to a zoonotic character, this food borne parasitosis represents important public health hazard as well as source of economic losses in animal husbandry.

The percentage of meat borne infections of the total number of toxoplasmosis patients can be estimated between 30% and 63% in Europe (2). Along with sheep, pigs are the species mostly associated with human infection (5). Because of the constant increase in demand for pork, stress on production rises worldwide. In the last century pork production underwent two major changes. First intensive farming systems have been developed in order to raise the number of animals produced and in the same time, prevent large outbreaks of infectious diseases (6). Though efficient, this system contributes little to animal welfare. This was the reason why, at the beginning of new century, animal friendly pig farming methods gained on popularity. Nowadays three major pig farming systems are recognized in the World: intensive, free-range (outdoor) and organic farming (6). At intensive farms animals are kept in strictly confined conditions, floors are made either out of concrete or wooden slates for easier and faster cleaning. Liquid or hard food is distributed automatically from food silos or by farm workers. Pigs are separated in fattening categories either per building or per batch, from where all animals are evacuated in the same time (all in all out system). Since 2013 EU banned the use of gestation crates in farm maternities ("sow stalls") for more than 4 weeks per gestation period, and reintroduced straw as bedding in sow pens (7). In these conditions, animals can express their natural behaviour, like foraging and nesting. Amongst intensive farms throughout Europe, a great variety of containment levels can be observed, from strictly confined breeding, up to closed farms, without regular rodent control or food storage protection. In contrast, both organic and outdoor pigs have access to natural light and weather conditions. In outdoor farms, animals are bred in semi-confined

spaces with outside access close to farm house. Sows are still inseminated artificially, but give birth in huts (animal caging is strictly forbidden) and piglets are taken to indoor facilities after four weeks, for the weaning period of one month. Straw and earth are used for bedding and animals are fed by farm workers. Some outdoor farms do not practice all-in all-out system, rather having the animals of different age categories in the same pens all the time. Based on national and regional histories of organic pig production, diverse climatic conditions and final product for which animals are reared, different housing systems are used for keeping sows, piglets and fattening pigs in organic farms of European countries (8). European union regulated organic pig breeding by Council Directives (7, 9). These directives provide very general recommendations, requiring only that pigs on organic farms have outside access. Type of the outside space, bedding, if outside pens are roofed or not, entirely depends on national and regional interpretations. In the UK for example, a voluntary industry code requires that free-range pigs have permanent access to pasture, while in Sweden this period is restricted to 6 months per year. They must be born and reared outside (without gestation or farrowing crates). In some countries sows are at pasture throughout all stages of pregnancy and lactation. In others, most sows are housed indoors during lactating. Mixed indoor and outdoor housing systems can also be found (8). For organically bred pigs, the use of medications and food additives is strictly controlled or is completely banned. For some pig breeds, even the artificial insemination is restricted to a minimum in order to enable better follow of animal origin.

So far, there is no routine surveillance of individual animals destined for human consumption nowhere in the World, and screening for *T. gondii* infection at slaughter does not exist in any country (10). Therefore, it remains unknown how animals get infected, what are the endemic areas, percentage of infected animals present at the world food markets or the exact role their meat plays in overall *T. gondii* epidemiology of humans.

Biology and epidemiology

Life cycle

T. gondii life cycle can involve all mammals and birds as intermediate hosts (propagation by clonal division) and members of the *Felidae* family as definitive hosts (sexual reproduction). One of the cycle characteristics is that parasite does not need definitive host in order to complete the life cycle (11). It can spread among intermediate hosts by predation or scavenging and replicate clonally, without a need for sexual reproduction. This characteristic is a basis for clonal structure of *T. gondii* population.

Oocysts are environmentally resistant forms, excreted by felids, and can be found on contaminated vegetables, fruits, in soil, all types of water and even in organisms that filter liquids for nutrients (oysters for example) (11-15). The ingestion of food or water contaminated with oocysts or

undercooked meat containing tissue cysts are the two major routes of postnatal transmission of *T. gondii* (16). After infection with any life stage, in new hosts, parasites transform in proliferative stage – tachyzoites, which are distributed throughout the body by circulation. Upon entering of tachyzoites in cells, they multiply by clonal division, causing acute toxoplasmosis. Until today, the factors that influence the encystation and conversion of tachyzoites to quiescent forms – bradyzoites, are not entirely described (17). When parasites interconvert under the influence of different host immune factors, bradyzoites form tissue cysts inside of parasitic vacuole. Tissue cysts persist for a long time, perhaps for the life of the host (18, 19). It has been hypothesised that tissue cysts rupture occasionally when released bradyzoites are killed in immunocompetent hosts or that immune system succeeds to limit the cysts by calcification, but so far no real proof has been shown. The tachyzoite – bradyzoite conversion depends mainly upon the immune status of the host and species-specific parasitic virulence. While for one animal species specific strain can be very pathogen, in another it produces mild or no clinical symptoms (20).

When felids prey on animals infected with *T. gondii*, sexual replication starts in intestines, as in all coccidian parasites. In cats, the bradyzoite - induced cycle is the most efficient, causing nearly all animals to shed oocysts, while less than 30% excrete parasites, when fed with tachyzoites or oocysts (21). Unlike many other *Coccidia*, oocysts of *T. gondii* are less infective and pathogenic in the definitive (cat) as compared with intermediate hosts (mice, pigs, humans, etc.) (18). Five morphologically distinct asexual forms occur in feline enterocytes, leading to a production of encapsulated zygote – oocyst. Once shed with faeces in nature, oocysts sporulate within 2-10 days depending on environmental conditions and become infectious for animals and humans (22). Oocyst walls of *Toxoplasma* have a rigid bilayer, dissolve in organic solvents, and contain a complex set of triglycerides, β -1,3-glucan and proteins cross-linked by dityrosines, building a waxy coat of acid-fast lipids that make them resistant to environmental stress (23). Sporulated oocysts can survive in water frozen at -10°C for 54 months, on +4 – 20°C for 54 months, whereas temperatures above 30 degrees shorten their survival considerably to 32 days (14, 24). In arid areas survival period of oocysts shortens considerably after 100 days, whereas damp and moist surfaces favour oocysts survival for as long as two years (25).

Strains and spread-out

Although capable of infecting almost all warm-blooded animals, the genus *Toxoplasma* have been classified with only one species (26, 27). While all isolates are morphologically similar, molecular analyses discovered high clonal structure (28). In first, species was characterized in three major lineages, because of their predominant presence in Europe and North America where first strains were isolated (29). Later with discoveries of strains in South America, Africa, and Asia a

higher frequency of non-clonal strains was revealed (atypical and recombinant), indicating that the population structure is more diverse than previously thought (4). Some of North American strains showed high genetic specificities that allowed formation of fourth clonal lineage (30-32). All clonal lineages show high similarity throughout their multilocus genome, with sequences highly specific for each group, and minor differences between strains of the same class (28). Interestingly, genetic bottleneck of these lineages happened around 10,000 years ago and after that they spread rapidly in Euro-Asia (33). Curiously, first pigs were domesticated in Middle-East before 13,000 years and were brought to Europe around 10,000 years ago, as well, along with domesticated ruminants (34).

Two major genotyping methods for *Toxoplasma gondii* have been developed. Ajzenberg, Banuls (35) described microsatellite method for fine distinction of strains. In 2006 Restriction Fragment-Length-Polymorphism (RFLP) was developed based on bi-allelic polymorphisms (36). Combining two methods in 2012, after analysis of 950 strains collected throughout the World, based on genetic origin, six major clads with 15 haplo-groups were constructed (28). More clonal and genetically similar strains originate from parts of the world where diversity of feline species and their numbers are not high – Europe, North America and Asia. Continents like South America and Africa, with larger numbers of felids, provide higher possibility for sexual reproduction of parasite and consequently higher genetic diversity (28). Though the new systematisation of strains is more precise, refined and offers explanations about strain origins; it is complicated for a routine basis because of time and costs. Moreover, it would mean reanalysis of all strains isolated from pigs so far, which is not an aim of this study.

In rapports across the world, pigs can be infected with various strains (Table 1) depending of their origin. As in other species, atypical strains can be found in pigs originating from South America and Africa. “China type” is characteristic in East Asia, while types I, II and III are dominant in Europe and North America. In Italy, in 2015 (37) atypical strain was isolated for the first time, but predominantly type II is present. Interestingly, live parasites were isolated not only from animal carcasses and different tissues, but also from meat products like sausages in Brazil (38) and cured hams in Spain (39).

Nowadays people travel more frequently; food and products are exchanged more quickly, which gives a parasite an opportunity for larger distribution throughout the world. Therefore, when describing the presence of *T. gondii* in different human populations it is necessary to take into account the culture and social background of studied groups. Ajzenberg, Collinet (40) described more genetically similar strains type II in French rural populations than those found in urban communities. Moreover, analysis of genetic similarities between strains isolated from locally bred animals destined for consumption, and human *Toxoplasma* cases proved the influence of locally made and consumed

food. The strain variability is consequence of different food sources as well as people travel opportunities.

Diagnostic

Indirect

Serological methods are the most commonly used for the diagnostic of *T. gondii* in animals and humans. Sabin Feldman dye test has been a gold standard for *T. gondii* specific antibody detection for years. Since the live parasites are used for agglutination reaction in dye tests, only reference laboratories can perform this analysis (41).

Desmonts and Remington (42) described Modified Agglutination Test (MAT), widely used for detection of *T. gondii* specific antibodies ever since. MAT is a species independent assay, which has been test of choice for investigation of serologic status of cattle (41), pigs (43-45), sheep (46, 47), chickens (48, 49), dogs and cats (50, 51), as well as humans (52, 53). Recently MAT has been used for detection of antibodies in meat destined for human consumption as well (54). Antigen for MAT consists out of whole parasites, fixed in formalin. In order to produce high quantities of antigen, Desmonts and Remington inoculated mice intra-peritoneally with mouse TG 180 sarcoma cells infected with *T. gondii* Rh strain parasites (type I). This method enabled collection of ten times more parasites in comparison with simple passage through animals (42). As described in their article, two major requirements need to be fulfilled in order to obtain antigen with satisfactory quality: i) intra-peritoneal exudate needs to be harvested when almost all sarcoma cells are heavily infected, close to disruption and a great number of free parasites that appear morphologically normal (alive) are observed; ii) mice should be inoculated between 48 - 72h before their exudate is collected. These two requirements are delicate to achieve resulting in careful production of antigen in only few laboratories. There are two major disadvantages of MAT: i) subjectivity - readings of the test results depend upon the examiner; ii) antigen production is restricted to specialized laboratories.

Main advantages of MAT are test's simplicity, species- independency, and possibility to use tissue fluids other than sera (11, 54). MAT has relatively low sensitivity at the beginning of infection and subjectivity of result reads. In the same time as MAT (1980), different immunological tests for detection of anti - *T. gondii* antibodies in humans were developed in order to standardize the interpretation of results (55-59). In contrast to MAT, these tests used different proteins from *T. gondii* surface (both tachyzoites and bradyzoites) as markers. Characteristic of these tests is their simplicity (IHAT, LAT) and high specificity and sensitivity (IFAT and ELISA). Nevertheless, IHAT and LAT lack sensibility, whereas IFAT and ELISA need species - specific conjugates that are not

always readily available for all analysed species (11). In 2013 comparison was made between IFAT, TgSAG1-ELISA (using native affinity-purified P30 (SAG1) *T. gondii* tachyzoite surface antigen) and Western blot (with tachyzoites lysate), on the samples from indoor and outdoor pigs (60). The estimated sensitivity of TgSAG1-ELISA on sera reached 93.1% and 98.8% in indoor and outdoor animals, respectively. In the last 20 years, various tests for diagnostic of specific anti- *T. gondii* antibodies in pigs were trialled (Table 1). When compared on pig sera, all ELISA tests showed sensitivities and specificities similar or higher than MAT, especially in experimental conditions. In 2012 new, bead based assay (BBA), showed consistent sensitivity and specificity as well as high concordance to commercial and in house ELISA tests (61) (Table 1). Though reliable and with high sensitivities and specificities these tests performed poorly when tissue fluids, other than sera, were used (Table 1). In 2012 (62), detection of anti *T. gondii* antibodies in experimentally infected pigs by MAT and ELISA was described. Both tests performed on sera, had 100% specificities and sensitivities, when mouse bioassay results served as standard, but sensitivity of ELISA dropped to 71.4% when heart fluid was analysed (62). This is especially important in studies, having in mind that in many slaughterhouses blood is collected through empty needle system and cannot be readily available from individual animals.

Epidemiology in animals

Wild animals

Toxoplasma gondii was found in almost all warm-blooded species of wild animals in Europe (Table 2 and 3). Parasite strains were isolated from majority of wild game species and migratory, as well as sedentary birds, throughout the whole continent. The lowest seroprevalence was found in mountain hares from Finland, and highest in red foxes from Germany (63, 64). In three raptor species from Spain, seroprevalence of 100% was found but the number of tested animals was low (4- *Lutra lutra*; 6 – *Mustela putorius*; 4 – *Genetta genetta*) (65) (Table 2). Negative seroprevalence were found in several species of rodents, and birds throughout France, Spain, Germany and deer from Belgium with low numbers of samples as well (Table 2). Herbivores are infected in lesser extent with *T. gondii*, up to 40%, throughout the whole continent (Table 2), irrespective to carnivores whose seroprevalence is above 35% (Table 2). In the same time prevalence in omnivores - wild boars, ranges from 5.2% in Northern Greece to 39.6% in Tatra mountains of Czech Republic. Wild boar seroprevalence reported from East and South-East European countries are considerably lower than investigations from the remaining part of continent. Interestingly when prevalence in two populations of foxes on islands of north England (Scotland) and Ireland were investigated, a significant difference of almost 50% was observed (6 and 55.8%, respectively) (66, 67). In large game species, the highest prevalence of 52% was found in Belgium and 50% in Czech Republic, while in south of Europe (Spain, Italy) the

prevalence are low between 13% in Roe deer and 25% in Fallow deer (Table 2). In France, the prevalence in deer varies from 17% - Red deer up to 60% - Roe deer, depending on region where samples were collected (68). In subfamily *Caprinae* (Mouflon, Chamois, Spanish Ibex, Barbary sheep) an altitude influences intensity of *T. gondii* infection. Thirty three point three per cent of Spanish ibex - living in plains and valleys of Spain are infected, in contrast to 5% of Chamois – found on steep slopes of Italian Alps (Table 2). In wild rabbits the seroprevalence is the same throughout the continent (around 10%), which is interestingly opposite to percentage of positive lynx (around 80%), foxes (around 60%) and wild cats (50%) who are their main predators. Birds preying on small rodents have higher possibility for infection than other species, which is proven in research from Spain, France and Italy (Table 2). In Spain extensive study of *T. gondii* seroprevalence in stranded dolphins was done in 2004 (69), and infection rate from 4% in Common to 36% in striped dolphins was found. Seroprevalence analysis goes to show that wild animals in and around Europe are in constant contact with *T. gondii*. Large number of strains has been isolated all over Europe from variety of wild animals (Table 2), and analysis of found genotypes prove that European wild life represents a reservoir for three clonal types of *T. gondii*, with predominance for types II and III.

Domestic animals

In Europe, during the last 15 years, seroprevalence of *Toxoplasma gondii* in majority of animal productions is well studied (Table 3). Sampling was mainly done in slaughterhouses. Data on origin of the animals was collected and farm prevalence was calculated. Although there were indications that in north European countries seroprevalence is negligible because of different climatic conditions, the research from Poland (goats, cattle), Sweden (horses) and Lithuania (sheep) prove the parasite's presence in these areas (43, 70-72). Relatively high percentage of seropositive cats is remarkable throughout the whole continent (73-76). In all countries small ruminants are highly infected (Table 3). Sheep and goats main nutrition is pasture where they can be contaminated by sporulated oocysts shed by cats. The prevalence varies from 84.5% in Serbia to 27.8% in Netherlands (77, 78). The higher prevalence in sheep is remarkable in south European countries, while it drops on North (Table 3). In horses, high prevalence was reported in Iberian Peninsula and Romania, but no strain was isolated so far (Table 3). In the south of Europe high prevalence were found for cattle, with the exception of Portugal and France. In middle and north European countries prevalence is considerably lower. The highest reported seroprevalence in cattle was from Spain (83.3%) and the lowest from Portugal and France (7.5%) (79-81). Data on presence of *T. gondii* in poultry in Europe are scarce. Only three studies reported seroprevalence around 25% in chickens from Portugal and Austria, 25.2% in geese, 20.2% in turkeys and 5.7% in ducks from Germany (82-85).

Pigs

Survey of *T. gondii* in pigs raised and consumed in Europe was conducted in 14 countries for the last 15 years. All age categories were analysed (piglets, fattening pigs, reproduction animals). Majority of studies used samples collected in the slaughterhouse, so the data for farm prevalence lack in 11 out of 18 studies (Table 4). In other studies, the highest farm seroprevalence was calculated in Spain (85%) and the lowest in Netherlands on intensive farms (4%) (86, 87). In Spain absence of rodent control increases the chance of *T. gondii* infection of pigs almost 2 times, while contact with cats elevates the risk between 3 and 11 times (Table 4). In Netherlands the main reason for such a low prevalence on intensive farms is high level of containment (16 times higher risk for organic and free-range farms (87). Out of five studies, that reported different prevalence between intensive and outdoor farming, four reported significant differences between two breeding methods, and only Berger-Schoch, Bernet (88) had contradictory results in Switzerland. The highest overall seroprevalence of 28.9% was recorded in Serbia and lowest of 2.16% in Slovakia (78, 89). The lowest seroprevalence in fattening pigs (0%) was recorded from Romanian intensive farms (90), but in this study, in house IFAT was used without previous standardization with control sera. Highest recorded prevalence in European pigs was shown by MAT in Austrian organic farms – 63.5% (45). Five studies showed significant difference in age prevalence. A study from Portugal, in 2013 showed higher percentage of positive piglets than fattening and reproduction animals (79). Since piglets are slaughtered in age younger than 120 days in Portugal, there is a good probability that detected antibodies are of maternal origin (91). Other risk factors for *T. gondii* infection of pigs throughout Europe were lack of rodent control, presence of cats on farms, cleaning practices, farm type and breeding practices (Table 4).

Direct detection of *Toxoplasma gondii*

Bioassay

In order to determine the presence of live, infectious *T. gondii* parasites in suspected tissues, bioassay in mice or cats is commonly used. While mouse assay is very adequate for determination of *T. gondii* pathogenicity to humans, sensitivity of the test depends upon the strain characteristics, used tissue and parasites concentration (92, 93). When concentration of specific anti *T. gondii* antibodies against type I is high, presence of parasites in mouse tissues can be detected within 24h p.i. Conversely, in case of a low infective dose with both types I or II, parasitaemia is undetectable in first 3-4 weeks p.i. (93). Therefore, for bioassay performed on animal tissues, period of four weeks is necessary for full development of infection in experimental animals –mice (54). In the same time, cats fed with only one tissue cyst can shed oocysts within 4-7 days (94). As well as in mice, time necessary for development of infection is strain-, dose- and cat age- dependent (95). In cat bioassay, quantity of examined materials is \approx 1kg per cat, during a course of 2-3 days, whereas only 100g can be digested

artificially and given per mouse, once. Therefore the sensitivity is much higher when cats are used for bioassay. On the other hand, SPF cats are expensive, and specialized animal facilities with trained personnel are needed. Because reduction and reuse of animals in this situation is not possible, mouse bioassay is more convenient throughout the world for isolation of infectious *T. gondii*, and propagation of the strains in laboratory conditions.

PCR based techniques

Although very specific, the sensitivity of bioassay is hard to determine and depends upon multiple factors. To overcome this obstacle, the development of new method for direct detection parasite was necessary. The first description of use of Polymerase Chain Reaction (PCR) in diagnostics of *T. gondii* infection in human amniotic fluids was described by Grover, Thulliez (96). Since then, the research on mapping parasite's genetic material advanced and first Homan, Vercammen (97) described 529- and then Grigg and Boothroyd (98) B1- highly repetitive fragments of *T. gondii* DNA. A non-coding 529 bp fragment that is repeated 200- to 300-fold in the genome of *Toxoplasma gondii* was able to detect 4 tachyzoites by standard PCR (97). Although 35- fold repetitive B1 fragment is 10 times less sensitive, restriction map of amplified DNA discriminated type I from II or III (98). Empirically (data not shown) B1 gene has been used mostly in diagnostic of acute human toxoplasmosis when there is already reasonable doubt of parasitic infection (e.g. high serology titers and detection of IgM). Whereas, 529 repetitive fragment was applied in epidemiological research of *T. gondii* in clinically healthy animals, with chronic infection, when parasitic burden in tissues was presumably low (e.g. low titer of IgG antibodies).

Strain isolation in Europe

Wild animals

In the last 15 years 378 strains of *T. gondii* were isolated from different samples of wild life throughout Europe. Almost 16% of these strains are not characterized yet. Only four strains found in birds in Italy and rabbits from UK are genotyped by RFLP as atypical strains (99, 100). Majority of the strains, 39.4%, belong to type II, and the rest of the strains are characterized as types I, III or clonal modifications of type II (Table 5). Carnivores (foxes, ferrets, wild cats, *Falconiform* birds, etc.) and wild boar were more infected than other wild animals. In north European countries surprisingly high number of strains was isolated from rabbits and wild hares, but no unusual strains were found. In the same time, contrary to the prevalence, small number of strains was isolated from wild ruminants. Looking at the Table 5, one can conclude that European wild life represents a great reservoir for *T. gondii*, and that most prevalent strains belong to three clonal lineages. Although well studied in west

and south EU countries, little is known about types of *T. gondii* prevalent in wild animals from east and south east of the continent, which are important reservoirs of wild life for Europe.

Pigs

Like in European wild life, majority of the isolates from pigs raised in Europe belong to three clonal lineages. Largest number of strains, 34.5%, belongs to type II isolated from Portugal, Italy, France and Slovakia (Table 6). Almost all strains belonging to types I and III, or clonal combinations were isolated from organic or free ranged animals.

Meat protection and inactivation of *T. gondii* in meat products

In 1965 prevalence of French population was 83%; 43.8% - 2003; and 37% in 2010 (constantly decreasing). The prevalence always varied substantially according to analysed region in time, population migrations and other complex epidemiological factors (40). A study of Nogareda, Le Strat (101) has predicted the prevalence of *T. gondii* among French pregnant women to be 27% until 2020. This prediction took into account majority of known risk factors for human infection. Sheep and pigs have traditionally been an important source of meat in many developing countries, and are the only species shown to regularly harbour the parasite (102). This is due to a high resistance of *T. gondii* in meat and delicatessen. In experimental conditions, bradyzoites in frozen meat survived for 7 days (103), but the temperatures above 58°C killed them after 9.5 minutes (104). When contaminated meat was exposed to pressure below 300-400 Mega Pascal (MP), 90% of parasites survived processing (105). In vacuum – packed, not salted goat meat parasites survived for over 6 weeks, while freezing of this meat at – 20°C, inactivated them after 3 hours (106).

One of the most important methods of curing the meat is chemical processing with sodium - chloride, and potassium- or sodium- lactate. Upon penetrating the meat, salts bind free water (water activity – a_w), which is essential for parasites survival. Without free water, elementary metabolic processes in parasites can not be performed, exchange of materials with environment is unable, thus organism's communication with its surrounding stops. Only 2% of NaCl or 1.4% potassium lactate inactivates parasites within 8 hours (103, 107). Salting in combination with thermic or pressure processing inactivate *T. gondii* within hours, while salted meat in vacuum protect parasites up to 14 days (106). Inappropriate or insufficient use of these processing techniques allow parasite to survive and stay infectious for animals and humans

Tables and Graphs:

Table 1 Literature analysis of published results on available tests for detection of anti-*Toxoplasma gondii* antibodies

The analysis of literature data shows high sensitivities and specificities for MAT and ELISA when pig sera were tested. In the same time ELISA showed better correlations with other indirect detection tests. The study of Forbes *et al* from 2012 showed higher sensitivity of MAT than ELISA when different tissue fluids are used for IgG analysis. Other studies proved similar sensitivity between MAT and ELISA, but higher specificity of ELISA than MAT. Other available serology tests showed lower values or no consistency with high variations between results.

Method	Sensitivity (%)	Specificity (%)	Experimental infection	Concordance to other test(s)	Number of pigs	Infectious dose	Year	DOI
	Compared to Bioassay	Compared to Bioassay						
MAT	100	87.5	YES	MAT-LAT-100%	24	10 oocysts	1996	Dubey, J. P.
LAT	100	100		MAT-IHAT-62.5%				Andrews, C. D.
IHAT	100	100		MAT-DT-87.5%				
MAT	100	100						Lind, P.
LAT	80.0	100	NO	MAT-LAT- 100%	24	1 oocysts	2013	Kwok, O. C.
IHAT	100	100		MAT-IHAT- 37.5%				Thulliez, P.
				MAT-DT-91.7%				Lunney, J. K.
IFAT	87.3	87.0	NO	IFAT-WB – 76.8%	297	NA	2013	10.1016/j.ijpara.2013.02.003
WB	93.5	77.2		IFAT-TgSAG1 – 86.9				
TgSAG1-ELISA	93.1	98.8		IFAT-PrioCHECK – 84.8				
ELISA PrioCHECK	98.9	92.7		WB-TgSAG1 – 83.8				
			NO	WB-PrioCHECK – 81.8	6	5000 oocysts	2015	10.1016/j.vetpar.2014.11.020
				TgSAG1-PrioCHECK – 93.9				
ELISA PrioCHECK	100	100						
Bead-Based Assay	85.5	91.3		BBA – ELISA – 85%				

MAT ELISA	85.7 88.6	94.6 98.0	NO		MAT-ELISA – 89.1%	274	NA	2005	10.1016/j.vetpar.2004.11.019
	Compared to LAT	Compared to LAT		2	LAT-TgSAG2 – 84.7%				
TgSAG2	75.0	96.3							
TgGRA7	90.6	85.2	NO	3	LAT-TgGRA7 – 88.1%	59	NA	2013	10.1128/CVI.00747-12
TgGRA14	81.2	92.6			LAT-TgGRA14 – 86.4%				
TgSAG1 ELISA	Compared to Immunoblot 100	Compared to Immunoblot 100	YES			12	10 ³ & 10 ⁴ –Rh 10 ³ & 10 ⁴ –ME49 10 ³ & 10 ⁴ –NED	2012	10.1016/j.vetpar.2012.04.014
	Compared to IFAT 92.8	Compared to IFAT 98.3	NO		IFAT-TgSAG1 ELISA – kappa 53%	602	NA		
ELISAs:									
PG10	100	100							
PG13	100	100	YES		NA	51	Gansu Jingtai strain	2013	10.1016/j.exppara.2013.09.019
PG18	100	100							
Bead-Based Assay	86.0 64.0 76.0 84.0	96.0 95.0 93.0 99.0		4	BBA-RIVM – kappa 68 BBA-Safepath – kappa 80 BBA-ID-VET – kappa 93 RIVM-Safepath – kappa 75 RIVM-ID-VET – kappa 72 Safepath-ID-VET – kappa 80	932	NA	2012	10.1186/1746-6148-8-36
RIVM-ELISA			NO						
Safepath –ELISA									
ID-VET-ELISA									
ELISA	sera 100	sera 100							
MAT	100	100	YES		NA	10	100 -1000 oocysts	2012	10.1016/j.vetpar.2012.06.040
	tissue fluids	tissue fluids	VEG strain						
ELISA	71.4	100							
MAT	100	100							
cELISA	88.1	94.5	NO		MAT-cELISA – 94.5%	861	NA	2011	973070 [pii]

	Compared to DT	Compared to DT 92.0							
Indirect ELISA	94.0	94.0	YES		191	10 ³ – 10 ⁴ tachyzoites	1997	10.1016/S0304-4017(97)00010-1	
Blocking ELISA (AgP30)	94.0		Rh strain	NA					

MAT – Modified agglutination test; LAT – latex agglutination test; IHAT – indirect hem-agglutination test; IFAT – Immuno-fluorescence antibody test; WB – western blot; ELISA – enzyme-linked immunosorbent assay; DT – Dye Test; Bioassay – mouse bioassay; Concordance has been calculated as mathematical concordance or Cohen’s kappa value.

Table 2 Seroprevalence of *Toxoplasma gondii* infection in European wild animals

Parasite is found in wild animals from both south and north countries in Europe. This shows that climate conditions do not impact *T. gondii* spread out as previously thought. The seroprevalence levels in various European countries show high contamination of wildlife and a size of parasitic reservoir for domestic animal and human contamination. Studies conducted in Europe mainly use MAT or ELISA test, therefore making the results comparable between countries.

Country	Animal species	Seroprevalence (%)	Total number	Number of isolates	Detection method	Author	Year	DOI
UK	...2.1.1.25 ferrets ;							
	...2.1.1.26 red foxes;	23.2	99	12				
		6.0	83	1				
		31.4	70	9	ELISA	Burrells, A., <i>et al</i>	2013	10.1017/s0031182013001169
	...2.1.1.27 polecats;	29.2	65	6				
		25.0	64	2				
	...2.1.1.28 minks; badgers; stoats	44.4	9	0				

Ireland	foxes		55.8	206	1	ELISA	Murphy, T. M., <i>et al</i>	2007	10.1016/j.vetpar.2007.02.017
Belgium	...2.1.1.29	roe deer;	52.1	73	1	ELISA	De Craeye, S., <i>et al</i>	2011	10.1016/j.vetpar.2010.12.016
	...2.1.1.30	bred fallow deer;	0	4	0				
			0	7	0				
		red deer; red fox	NA	304	57				
Sweeden	Red fox		38.0	221	NA	DAT	Jakubek, E.B., <i>et al</i>	2001	
	Lynx lynx		75.4	207	NA	DAT	Ryser-Degiorgis, M. P., <i>et al</i>	2006	10.7589/0090-3558-42.1.182
	Wild boar		38.0	1327	NA	ELISA	Wallander, C., <i>et al</i>	2014	10.1017/S0950268814002891
Finland	brown hares; mountain hares		12.1 4.0	116 99	14 4	ELISA	Jokelainen, P., <i>et al</i>	2011	10.7589/0090-3558-47.1.154
Latvia	Wild boar		33.2	606	NA	ELISA	Deksne, G. and Kirjusina M.	2012	10.1645/GE-3187.1
Germany	red fox; rodents		85.3 0	353	48 0	Immunoblot	Herrmann, D. C., <i>et al</i>	2012	10.1016/j.vetpar.2011.10.030
Czech Republic	...2.1.1.32	Sika deer	50.0	14	NA	IFAT	Bartova, E., <i>et al</i>	2008	10.1645/GE-1126R.1
			45.0	377					
	...2.1.1.33	Red deer	24.0	79					
			17.0	143					
	...2.1.1.34	Roe deer	9.0	105					

	Fallow deer								
	Mouflon								
	Wild boar		39.6	656	NA	ELISA	Racka, K., <i>et al</i>	2015	10.5604/12321966.1152071
Slovakia	Wild boar		8.1	320	NA	NA	Antolova, D., <i>et al</i>	2007	
Hungaria	Red fox		68.0	337	NA	DAT	Jakubek, E.B., <i>et al</i>	2006	10.1016/j.vetpar.2006.09.011
Greece	Wild boar		5.2	94	NA	ELISA	Touloudi, A., <i>et al</i>	2015	10.1136/vetreco-2014-000077
Italy	...2.1.1.35	Anas platyrhynchos							
	...2.1.1.36	Anas clypeata	11.8	17					
			18.2	11	3	NA	Mancianti, F., <i>et al</i>	2013	10.1645/12-34.1
	...2.1.1.37	Anas crecca	7.3	41					
			25.0	8					
	...2.1.1.38	Gallinago gallinago							
	Roe deer		13.0	207	NA	LAT	Gaffuri A., <i>et al</i>	200-	10.7589/0090-3558-42.3.685
	Chamois		5.0	108					
Spain	...2.1.1.39	Striped dolphin	11.1	36					
			50.0	4	NA	MAT	Cabezon, O., <i>et al</i>	2004	10.1645/ge-257r
			57.1	7					
	...2.1.1.40	Commo	0	9					

	n dolphin								
...2.1.1.41	Bottlen ose dolphin								
	Rissos's dilphin								
...2.1.1.42	red dear;								
...2.1.1.43	fallow deer;								
...2.1.1.44	roe deer	15.6 24.0 21.8	441 79 33						
...2.1.1.45	spanish ibex;	33.3 20	3 10	NA	MAT	Gauss, C.B.L., <i>et al</i>	2006	10.1016/j.vetpar.2 005.11.013	
...2.1.1.46	chamoi s	10 14.8	10 27						
...2.1.1.47	Barbary sheep								
...2.1.1.48	mouflo n								
Wild boar		38.4	507	NA	MAT	Gauss, C.B.L., <i>et al</i>	2005	10.1016/j.vetpar.2 005.04.023	
Lynx pardinus		81.5	27						
Felis silvestris		50.0	6						
Vulpes vulpes		64.7	102	NA	MAT	Sobrino, R., <i>et al</i>	2007	10.1016/j.vetpar.2 007.06.038	
Canis lupus		46.9	32						
Meles meles		70.3	37						

	Martes foina	85.0	20					
	Martes martes	100.0	4					
	Lutra lutra	100.0	6					
	Mustela putorius	100.0	4					
	Genetta genetta	61.9	21					
	Herpestes ichneumon	59.1	22					
	Falconiformes	23.8	610					
	Strigiformes	44	260	NA	MAT	Cabazon, O., <i>et al</i>	2011	10.1371/journal.pone.0029549
	Ciconiformes	11	156					
	Gruiformes	5.6	21					
	Capra pyrenaica hispanica	27.5	531	NA	ELISA	Garcia-Bocanegra, I., <i>et al</i>	2012	10.1016/j.tvjl.2010.11.011
	Lepus granatensis	11.4	298	NA	MAT	Fernandez-Aguilar, X., <i>et al</i>	2013	10.1016/j.vetpar.2013.01.061
Portugal	pigeon	4.6	695	12	DAT	Waap, H., <i>et al</i>	2008	10.1016/j.vetpar.2008.07.017
	Wild boar	20.6	97	NA	MAT	Coelho, C., <i>et al</i>	2014	10.7589/0090-3558-44.1.172
France - Corsica	wild boar	0.55	1399	NA	MAT	Richomme, C., <i>et al</i>	2010	10.1017/S0950268810000117
	wild boar	17.6	148	21	MAT	Richomme, C., <i>et al</i>	2009	10.1016/j.vetpar.2009.06.014
France	Apodemus sp;	4.4	92	0				
	C. glareolus; Microtus	4.7	64	0				
	agrestis; Micromys	0	26	0				
	minutus;	0	4	0	MAT	Afonso, E. <i>et al</i>	2007	
	Sorex sp.;	60	5	0				
	Arvicola terrestris;	22.2	9	0				
	Talpa europaea	38.9	18	0				
	...2.1.1.49	79	14	0				
	Buteo buteo	50	12	0	MAT	Aubert, D. <i>et al</i>	2008	10.7589/0090-3558-44.1.172
		11	18	1				

...2.1.1.50	Strix aluco	0	8	0					
		0	1	0					
...2.1.1.51	Tyto alba								
Falco tinnunculus									
Accipiter nisus									
Capreolus capreolus		60	60	12					
Ovis gmelini		23	31	1					
Cervus elapus		17	24	1					
Dama dama		25	4	0	MAT	Aubert, D. <i>et al</i>	2010	10.1016/j.vetpar.2010.03.033	
Lepus europaeus		9	23	0					
Vulpes vulpes		74	19	9					
Anas platyrhynchos		50	4	1					
Rupicapra rupicapra		16.8	101						
Ovis gmelini musimon		14.7	143	NA	MAT	Gotteland, C., <i>et al</i>	2014	10.1089/vbz.2013.1304	
Capreolus capreolus		43.7	1155						

Table 3 Seroprevalence of *Toxoplasma gondii* in domestic animals of Europe

The table shows magnitude of parasite presence on the “old continent”. Mostly the farm animals were studied showing the potential levels of meat contamination present on European markets. Interestingly cat prevalence in various European countries do not vary and cat population is more or less infected evenly throughout the continent. Main tests for sero analyses used are MAT and ELISA making the results comparable. Still, mainly the researches are based on individual numbers of animals per country or region, rather than a farm. If there is one tissue cysts or oocysts on the farm all animals have the same probability to become infected, and we still know little about farm contamination levels.

Country	Animal species	Number of farms	Farm prevalence	Total number	Sero - prevalence (%)	Number of isolates	Detection method	Author	Year	DOI
Portugal	Chickens	NA	NA	225	27.1	16	MAT	Dubey, J. P., <i>et al</i>	2006	10.1645/GE-652R.1
	Sheep	160	57.5	1467	83.0	NA	MAT	Sousa, S., <i>et al</i>	2009	10.1111/j.1863-2378.2008.01187.x
	Horses	NA	NA	173	13.3	NA	MAT	Lopes, A. P., <i>et al</i>	2013	10.1186/1756-3305-6-178
	Cattle	NA	NA	161	7.5	NA	MAT	Lopes, A. P., <i>et al</i>	2013	10.1016/j.vetpar.2012.12.001
	Goats	NA	NA	184	18.5	NA	MAT	Lopes, A. P., <i>et al</i>	2013	10.1016/j.vetpar.2013.12.017
	Cats	NA	NA	215	20.5	NA	MAT	Esteves, F., <i>et al</i>	2014	10.1016/j.vetpar.2013.12.017
Spain	Cats	NA	NA	220	45	NA	MAT	Gauss, C. B., <i>et al</i>	2003	10.1645/ge-114
	Cats	NA	NA	585	32.3	NA	IFAT	Miro, G., <i>et al</i>	2004	10.1016/j.vetpar.2004.08.015
	Horses	327	14.7	454	10.8	NA	MAT	Garcia-Bocanegra, I., <i>et al</i>	2012	10.1016/j.parint.2012.02.003
	Mules	46	23.9	80	15.0	NA				
	Donkeys	47	34.0	82	25.6	NA				
	Cattle	72	100	504	83.3	NA	ELISA	Garcia-Bocanegra, I., <i>et al</i>	2013	10.1645/12-27.1
	Sheep	72	84.7	403	49.3	NA				
	Goats	72	72.2	494	25.1	NA				
France	Cattle	24	87.5	1329	7.8	NA	MAT	Gilot-Fromont, E., <i>et al</i>	2009	10.1016/j.vetpar.2008.12.004

	Lambs	NA	NA	83	15.0	34					10.1016/j.ijpara.2009.06.009
	Adult sheep			343	81.0	14	MAT	Halos, L., <i>et al</i>	2010		
Italy - Sardinia	Sheep	22	100	1043	51.3	NA	ELISA	Natale, A., <i>et al</i>	2007		
Italy - Sicily	Sheep	62	87	1961	49.9	NA	ELISA	Vesco, G., <i>et al</i>	2007	10.1016/j.vetpar.2007.02.019	
Italy	Sheep	33	97.0	630	33.3	NA	IFAT	Cenci-Goga, B. T., <i>et al</i>	2013	10.1186/1746-6148-9-25	
	Goat	6	100	127	60.6	10	MAT	Mancianti, F., <i>et al</i>	2013	10.1155/2013/905326	
Italy - Alpes	Goats	42	96.6	474	41.7	NA	IFAT	Gazzonis, A. L., <i>et al</i>	2015	10.5604/12321966.1141370	
	Sheep	42	87.5	502	59.3	NA					
Italy	Horses	60	NA	643	3.0	NA	IFAT	Bartova, E., <i>et al</i>	2015	10.14411/fp.2015.043	
Greece	Sheep	69	98.6	1501	48.6	NA	ELISA	Tzanidakis, N., <i>et al</i>	2012	10.1016/j.vetpar.2012.07.020	
	Goats			541	30.7	NA					
Turkey	Sheep	NA	NA	181	31	NA	ELISA	Oncel, T. and Vural, G.	2006		
	Cattle	NA	NA	132	56.06	NA	SFDT	Yagci Yucel, S., <i>et al</i>	2014	10.5152/tpd.2014.3454	
Switzerland	Cattle	NA	NA	406	45.6	NA	ELISA	Berger-Schoch, A. E., <i>et al</i>	2011	10.1111/j.1863-2378.2011.01395.x	
	Sheep	NA	NA	250	61.6	NA					
Austria	Chickens	11	100	830	36.3	67	MAT	Dubey, J. P., <i>et al</i>	2005	10.1016/j.vetpar.2005.06.006	
	Dogs	NA	NA	1770	26.0	NA	IFAT	Wanha, K., <i>et al</i>	2005	10.1016/j.vetpar.2004.11.027	
Serbia	Cattle	NA	NA	611	76.3	NA	MAT	Klun, I., <i>et al</i>	2006	10.1016/j.vetpar.2005.08.010	
	Sheep	NA	NA	511	84.5	NA					
	Pig	NA	NA	605	28.9	NA					
	Goats	143	84.6	431	73.3	NA	MAT	Djokic, V., <i>et al</i>	2014	10.4081/gh.2014.37	
Romania	Cats	NA	NA	236	47	NA	ELISA and MAT	Gyorke, A., <i>et al</i>	2011	10.1016/j.prevetmed.2011.07.015	
	Goats	NA	NA	735	52.8	NA	ELISA	Iovu, A., <i>et al</i>	2012	10.1016/j.vetpar.2011.11.062	
	Horses	NA	NA	82	39	0	ELISA	Pastiu, A. I., <i>et al</i>	2015	10.1016/j.vetpar.2015.07.024	
Belgium	Cats	NA	NA	346	70.2	NA	NA	Dorny, P., <i>et al</i>	2002		
Netherlands	Sheep	11	NA	1179	27.8	NA	ELISA	Opsteegh, M., <i>et al</i>	2010	10.1016/j.prevetmed.2010.	

	Calves	NA	NA	357	3.1	NA				06.009
	Adult cattle	NA	NA	638	25.0	NA	ELISA	Opsteegh, M., <i>et al</i>	2011	10.1016/j.ijpara.2010.10.006
Germany	Ducks	61	32.8	2534	5.7	NA	ELISA	Maksimov, P., <i>et al</i>	2011	10.1016/j.vetpar.2011.05.049
	Geese	13	84.6	373	25.2	NA				
	Turkey	14	100	1913	20.2	NA	KELA	Koethe, M., <i>et al</i>	2011	10.1016/j.vetpar.2011.03.036
Czech Republic	Sheep	9	100	547	59	NA	ELISA	Bartova, E., <i>et al</i>	2009	10.1016/j.vetpar.2008.12.022
Slovakia	Goat	1	100 ☺	116	56.9	NA	ELISA	Cobadiova, A., <i>et al</i>	2013	10.2478/s11686-013-0171-5
Poland	Goats	49	100	1060	NA	NA	ELISA	Czopowicz, M., <i>et al</i>	2011	10.1016/j.vetpar.2011.01.039 973070 [pii]
	Cattle	NA	NA	865	14.6	NA	cELISA	Sroka, J., <i>et al</i>	2011	
Sweeden	Horses	NA	NA	414	0.5	NA	DAT	Jakubek, E. B., <i>et al</i>	2006	10.1016/j.vetpar.2006.02.002
Lithuania	Sheep	NA	NA	354	42.1	NA	ELISA	Stimbirys, A., <i>et al</i>	2007	

Table 4 Seroprevalence of *Toxoplasma gondii* in European pigs

Infection of pigs in Europe is high. This table proves high parasitic burden in pigs throughout the continent. Older age categories, finishing farms and presence of cats are identified as major risk factors. Still individual prevalence are investigated more than farm contamination levels, therefore the data lack for on farm contamination.

Country	Category	Number of farms	Number of tested animals	Test	Number of isolated strains	Farm prevalence	Risk factors	Author	Year	DOI
Portugal	fattening	small farms in semi-intensive management	333	MAT	15	NA	NA	de Sousa, S. A., D., <i>et al</i>	2006	10.1016/j.vetpar.2005.08.012
	21 piglets 180 fattening 53 sows	14	254	MAT	NA	NA	age	Lopes, A. P., <i>et al</i>	2013	10.1016/j.vetpar.2012.12.001
Spain	1400 adult 1570 fattening	100	2970	MAT	NA	85.0	region, 2.9 X age, 1.9X lack of rodent control, 1.6X cats	Garcia-Bocanegra, I., <i>et al</i>	2010	10.1016/j.parint.2010.06.001
Spain - Catalonia	440 piglets 440 fattening pigs 322 sows	23	1202	MAT	NA	NA	age, 11.3X presence of cats, 0.5X outdoor facilities	Garcia-Bocanegra, I., <i>et al</i>	2010	10.1016/j.rvsc.2010.01.017
France	Pigs from intensive farms Pigs from outdoor farms	752	1549	MAT	41	NA	Age, farming type	Djokic, V., <i>et al</i>	2016	
Italy	fattening	10	960	IFAT	NA	16.1	cleaning practices farm management	Veronesi <i>et al</i>	2011	
Serbia	468 fattening 20 sows	sampled in abattoir	488	MAT	16	NA	41X age, 15X small farms	Klun, I., <i>et al</i>	2011	10.1186/1297-9716-42-17
Romania	fattening sows	sampled in abattoir	3595	IFAT	NA	NA	farm type, presence of cats	Pastiu., <i>et al</i>	2013	

	outdoor pigs									
Switzerland	120 adults 50 fattening intensive 100 free-ranging	sampled in abattoir	270	ELISA	NA	NA	age	Berger-Schoch, A. E., <i>et al</i>	2011	10.1111/j.1863- 2378.2011.01395.x
	sows	58	108	ELISA	3 strains	37.3	NA	Basso, W., <i>et al</i>	2014	10.1016/j.parint.2014 .11.017
Austria	70 intensive farms 3 organic farms	73	1368	MAT	NA	23.3;	NA	Steinparzer, R., <i>et al</i>	2015	10.1111/zph.12122
Slovakia	fattening	sampled in abattoir	970	ELISA	Types I and II	NA	age	Turcekova, L. A., D., <i>et al</i>	2013	10.2478/s11686-013- 0154-6
Poland	fattening	sampled in abattoir	861	ELISA	NA	NA	NA	Sroka, J., <i>et al</i>	2011	973070 [pii]
Germany	sows from 16% pedigree breeding 65% piglet production 19% farro to finish	94	2041	ELISA	NA	overall 68	1.2X age	Damriyasa, I. M., <i>et al</i>	2004	10.1016/j.vetpar.200 4.07.016
	sows	75	1500	ELISA	NA	56	NA	Damriyasa, I. M. and Bauer, C.	2005	
Netherlands	402 organic 178 free-range 265 intensive	40 organic 9 free-range 24 intensive	845	ELISA	NA	25 33 4	16X free-range	van der Giessen, J. F., M., <i>et al</i>	2007	10.1016/j.vetpar.200 7.06.009
Sweden	fattening sows	sampled in 10 abattoirs	807	ELISA	NA	NA	NA	Lunden, A., <i>et al</i>	2002	
Latvia	803 intensive pigs 606 farmed wild boar	23 slaughterhouses	1409	ELISA	NA	NA	17.6X farming	Deksne, G. and Kirjusina, M.	2013	10.1645/GE-3187.1

Seroprevalence

01 – 5%

5.1 – 10%

10.1 – 15%

15.1 – 20%

20.1 – 25%

25.1 – 30%

Table 5 Strains of *Toxoplasma gondii* found in wildlife throughout the Europe

As wildlife is considered a reservoir for parasites, European studies show that old continent, with small numbers of felis, is still sentinel for strains of type I, II and III. The number of strains isolated from various species proves that the parasitic burden is high. Even in migratory species of birds the most prevalent are “local” European strains.

Country	Animal species	Total number	Number of isolates	Detection method	Strain type	Author	Year	DOI
UK	...2.1.1.52 ferrets;				type II	Burrells, A., <i>et al</i>	2013	10.1017/s0031182013001169
	...2.1.1.53 red foxes;	99 83	12 1	PCR, RFLP	type II			
	...2.1.1.54 polecat s;	70 65 64 9	9 6 2 0		types I and III, type II			
	...2.1.1.55 minks;				type II			
	badgers; stoats							
	rabbit	57	39	PCR	10 type I, 6 type II, 7 type III, 1 atypical	Hughes, J. M., <i>et al</i>	2008	10.1016/j.exppara.2008.07.011
Belgium	...2.1.1.60 roe deer;			PCR, RFLP	NA	De Craeye, S., <i>et al</i>	2011	10.1016/j.vetpar.2010.12.016
	...2.1.1.61 bred fallow deer;	73 4 7 304	1 0 0 57		NA			
					NA			
	red deer; red fox				25 type II, 1 type III			
Sweeden	...2.1.1.62 Brown rat	49 86 12	0 0 0	PCR	NA	Backhans, A., <i>et al</i>	2012	10.1017/s0950268812002609

	...2.1.1.63	House mouse							
	...2.1.1.64	Yellow necked mouse							
Finland	brown hares; mountain hares	116 99	14 4	PCR, microsatelites	14 type II, 4 type II	Jokelainen, P., <i>et al</i>	2011	10.7589/0090-3558-47.1.154	
Germany	red fox; rodents	353	48 0	PCR, RFLP	13 type II, 4 type II clonal	Herrmann, D. C., <i>et al</i>	2012	10.1016/j.vetpar.2011.10.030	
	beavers; wildcats	6 12	2 4	PCR, RFLP	2 type II; 4 type II	Herrmann, D. C., <i>et al</i>	2013	10.1016/j.vetpar.2012.08.026	
Czech Republic/ Germany	wild house mouse	360	2	PCR	NA	Hurkova-Hofmannova, L., <i>et al</i>	2014	10.1645/13-255.1	
Czech Republic	red foxes;	152	2	PCR	NA	Hurkova, L. and Modry D.	2006	10.1016/j.vetpar.2006.01.005	
	martens;	61	3						
	badgers;	19	0						
	raccon dogs;	3	0						
	polecats;	2	0						
	ferrets;	2	0						
otter	1	0							
Austria	<i>C. glareolus</i> ;	40	0	PCR	NA	Schmidt, S., <i>et al</i>	2014	10.1089/vbz.2013.1504	
	<i>Apodemus flavicollis</i> ;	29	0						
	<i>Apodemus sylvaticus</i> ;	26	0						
	<i>Microtus arvalis</i>	15	0						
Switzerland	Wild boar	150	1	PCR	NA	Berger-Schoch, A. E., <i>et al</i>	2011	10.1016/j.vetpar.2010.11.046	
Italy	...2.1.1.65	roe deer;	121 105 94 22	3 17 19 0	PCR	NA	Ferroglia, E., <i>et al</i>	2014	10.1186/1756-3305-7-196

...2.1.1.66	wild boar;	13	0						
...2.1.1.67	red fox; alpine chamois; red deer								
	<i>Anas platyrhynchos</i>								
	<i>Anas clypeata</i>								
	<i>Anas crecca</i>	17							
...2.1.1.68	<i>Gallina go gallinago</i>	11 41 8	3	PCR	Atypical		Mancianti, F., et al	2013	10.1645/12-34.1

Spain	...2.1.1.69	wild boar;				2 type II, 2 type I, 3 PM I + III;			
	...2.1.1.70	red deer;	61	9		2Type II, 1 type III, 1 PM;			
	...2.1.1.71	fallow deer;	22	4		4 type II;			
	...2.1.1.71		21	10	PCR, RFLP	3 type II;			
	...2.1.1.71		12	3		1 type III, 4 type II;	Calero-Bernal, R., et al	2015	10.7589/2013-09-233
	...2.1.1.72	mouflon;	26	12		4 PM I+III, 5 type III, 2 type II			
	...2.1.1.72		41	21					
	...2.1.1.73	spanish ibex;							
	...2.1.1.74	red fox							
	<i>Buteo buteo;</i>		17	0					
	<i>Garrulus glandarius;</i>		23	5	PCR	NA	Darwich, L., et al	2012	10.1016/j.vetpar.2011.07.024
	<i>Gyps fulvus;</i>		105	1					
	<i>Milvus migrans;</i>		3	1					

	<i>Pica pica</i>	33	5					
Portugal	pigeon	695	12	microsatelites	9 type II, 2 type III, 1 type I	Waap, H., <i>et al</i>	2008	10.1016/j.vetpar.2008.07.017
France	wild boar	148	21	mouse bioassay, RFLP, microsatelites	21 type II	Richomme, C., <i>et al</i>	2009	10.1016/j.vetpar.2009.06.014
	<i>Buteo buteo</i>	14	0			Aubert, D. <i>et al</i>	2008	10.7589/0090-3558-44.1.172
	<i>Strix aluco</i>	12	0					
	<i>Tyto alba</i>	18	1	bioassay	Type II			
	<i>Falco tinnunculus</i>	8	0					
	<i>Accipiter nisus</i>	1	0					
	<i>Capreolus capreolus</i>	60	12		12 type II	Aubert, D. <i>et al</i>	2010	10.1016/j.vetpar.2010.03.033
	<i>Ovis gmelini</i>	31	1	Bioassay, RFLP, microsatelites	1 type II			
	<i>Cervus elapus</i>	24	1		1 type II			
	<i>Dama dama</i>	4	0					
	<i>Lepus europaeus</i>	23	0					
	<i>Vulpes vulpes</i>	19	9		9 type II			
	<i>Anas platyrhynchos</i>	4	1		1 type II			

Table 6 *Toxoplasma gondii* strains isolation from pigs in Europe

T. gondii is isolated mainly from pigs in south and central European countries. Genotyping of the strains shows that the predominant strain is type II, and that only in rare cases atypical strains can be present in pigs raised in Europe. Numbers of isolated strains, in individual studies, show high parasitic

Country	Pig type	Number of farms	Number of seropositives	Total number	Number of isolates	Detection method	Strain	Tissue	Author	year	DOI
Ireland	304 male and female	NA	14	304	13	PCR	NA	diaphragm	Halova, D., <i>et al</i>	2013	10.1111/j.1863-2378.2012.01514.x
Italy	organic, free-range	3	20	21	12	PCR, RFLP	3 type II, 5 type I/II, heart 3 type I		Bacci, C., <i>et al</i>	2015	10.1016/j.ijfoodmicro.2015.03.002
	intensive farms - sows	58	26	108	4	PCR	NA	placenta aborted fetuses	Basso, W., <i>et al</i>	2015	10.1016/j.parint.2014.11.017
Switzerland	120 adult 50 finishing 100 organic	minimum 135	NA	270	6	PCR, RFLP	1 type I/II	diaphragm	Berger-Schoch, A. E., <i>et al</i>	2011	10.1016/j.vetpar.2010.11.046
	100 fattening 100 sows	sampling in the abattoir	28	200	0	PCR	NA	brain	Wyss, R., <i>et al</i>	2000	
Serbia	468 fattening 20 sows	sampling in the abattoir	45	488	12	mouse bioassay	NA	blood cloth	Klun, I., <i>et al</i>	2011	10.1186/1297-9716-42-17
Slovakia	923 fattening 47 sows	36 farms 18 districts	21	970	21	PCR	18 type I, 3 type II	brain or muscle	Turcekova, L., <i>et al</i>	2013	10.2478/s11686-013-0154-6

Objectives

The aim of this study was to describe the prevalence of the parasite *Toxoplasma gondii* in pigs raised and slaughtered in France. When considering pork as still the most consumed meat in Europe and high risk burden for human infection with *T. gondii*, it is necessary to understand the routes of parasite emerging in pig production systems as well as to have sensitive methods to identify animals *T. gondii* carriers. Therefore, to investigate on farm risk factors responsible for the infection of pigs, seroprevalence, obtained by MAT, was compared to epidemiological data collected on intensive farms from west French region of Bretagne. The comparison between intensive and outdoor farming as a risk factor for *T. gondii* infection of pigs was analysed separately in a national survey conducted throughout eight major pork producing regions of France. Then, in order to evaluate the performance and usage of existing methods for diagnostics of infection and propose new and simple methods for standardized animal screening programs, the relationship between the presence of specific anti - *T. gondii* antibodies and live parasites in pork tissues was investigated. To evaluate the risk of human infection by consumption of seropositive pig meat, comparison between MAT results and direct parasitic isolation was analysed. The mouse bioassay, PCR and MC-PCR were competed to investigate the presence of parasites and compare the sensitivity and specificity of MAT. In cases when sera cannot be available sensitivity and specificity of cardiac and diaphragm fluids were analysed. Control of animals on farms and in slaughterhouses urged a need for an easy and cost effective method of antigen provision for serology analysis by MAT.

22 **1. Section 1:**

23 Article accepted for publication in Parasitology journal

24 ***Toxoplasma gondii* infection in pork produced in France**

25 DJOKIC V.^{1,5}, BLAGA R.², AUBERT D.³, DURAND B.⁴, PERRET C.¹, GEERS R.³, DUCRY T.¹,
26 VALLEE I.¹, DJURKOVIC DJAKOVIC O.⁵, MZABI A.³, VILLENA I.³, BOIREAU P.¹

27
28 ¹ Ecole Nationale Vétérinaire d'Alfort, ANSES, INRA, Université Paris-Est, Laboratoire de santé
29 animale de Maisons-Alfort, UMR BIPAR, Maisons-Alfort, France; Electronic address:
30 vitomir.djokic@anses.fr

31 ² Ecole Nationale Vétérinaire d'Alfort, ANSES, Université Paris-Est, UMR BIPAR, Maisons-Alfort,
32 France.

33 ³ Université Reims Champagne-Ardenne, Laboratoire de Parasitologie-Mycologie, EA 3800, SFR
34 CAP-SANTE, Centre National de Référence de la Toxoplasmose, Centre de Ressources Biologiques
35 Toxoplasma, Hôpital Maison Blanche, Reims, France.

36 ⁴ ANSES, INRA, Université Paris-Est, Laboratoire de santé animale de Maisons-Alfort, Epidemiology
37 unit, Maisons-Alfort, France

38 ⁵ Institute for Medical Research, National Reference Laboratory for Toxoplasmosis, University of
39 Belgrade, Dr. Subotića 4, P.O. Box 102, Belgrade 11129, Serbia

1.1. SUMMARY

The aim of this study was to assess the seroprevalence of the *T. gondii* parasite in pork produced in France, and to determine infection risk factors. An innovative survey was designed based on annual numbers of slaughtered pigs from intensive and outdoor farms in France. A total of 1,549 samples of cardiac fluids were collected from pig hearts to determine seroprevalence using a Modified Agglutination Test (MAT). Of those, 160 hearts were bio-assayed in mice to isolate live parasites. The overall seroprevalence among fattening pigs was 2.9%. The adjusted seroprevalence in pigs from intensive farms was 3.0%; the highest in sows (13.4%); 2.9% in fattening pigs, and 2.6% in piglets. Adjusted seroprevalence in fattening animals from outdoor farms was 6.3%. Strains were isolated from 41 animals and all were genotyped by Restriction Fragment Length Polymorphism (RFLP) as type II. Risk-factor analysis showed that the risk of infection was more than three times higher for outdoor pigs, and that sows' risk was almost five times higher than that of fattening animals. This study provides further evidence of extensive pork infection with *T. gondii* regardless of breeding systems, indicating that farm conditions are still insufficient to guarantee "Toxoplasma-free pork".

Key words:

Toxoplasma gondii, pork, intensive farms, outdoor farms, seroprevalence, parasite isolation

KEY FINDINGS

- This study reports on an innovative survey of *Toxoplasma gondii* infection in pigs from France, born and raised on intensive and outdoor farms.
- Seroprevalence, strain isolation and risk-factor analysis were assessed based on production systems, age categories and regions of slaughtering.
- The adjusted seroprevalence in pigs from intensive farms was 3.0%, highest in sows (13.4%), 2.9% in fattening pigs, and 2.6% in piglets. Outdoor farm seroprevalence in fattening pigs was 6.3%.
- Forty-one strains were isolated (25 from intensive and 16 from outdoor farms) and all strains were genotyped as type II.
- Risk-factor analysis showed an above three-fold (3.62) increased the risk of infection for pigs raised on outdoor farms ($P=0.004$), and that sows' risk of infection is nearly five times (4.63) higher than that of fattening animals ($P<0.001$).

- The study provides further evidence of extensive pork infection with *T. gondii* regardless of breeding systems, and that current zootechnical and hygienic measures are not sufficient enough to guarantee pork as Toxoplasma-free in these conditions. More detailed risk-factor analysis is necessary in order to provide specific recommendations for pig breeding production.

1.2. INTRODUCTION

With the widest range of mammalian hosts, including humans, *Toxoplasma gondii* is the most unrestricted parasite on Earth, infecting one third of the global human population (Torrey & Yolken, 2013). The organism has a complex life cycle that can involve all mammals and birds as intermediate hosts (by clonal division) and members of the Felidae family as definitive hosts (by reproduction). In immunocompetent individuals, *T. gondii* generally causes mild and self-limited infection. However, in categories of the population with a compromised immune system, such as foetuses and immunosuppressed individuals, the parasite may cause life-long infection of the nervous system and eyes, or life-threatening disease.

Human infection occurs through the ingestion of tissue cysts, present in contaminated meat, or oocysts (excreted by felids), via contaminated water, fruits and vegetables. A recent study has predicted the prevalence of *T. gondii* at 27% among pregnant French women until 2020 (Nogareda et al., 2014). Along with sheep, pigs are the species mostly associated with human infection (Esteban-Redondo et al., 1999). Consumption of infected undercooked meat or meat products is considered a major risk factor, especially in Europe, which has accounted for 30–63% of infections (Bobic et al., 1998; Cook et al., 2000; Tenter et al., 2000). Pigs have traditionally been an important source of meat in many developing countries, and are the only species shown to regularly harbour the parasite (Hill & Dubey, 2013). Bradyzoites in tissue cysts have been experimentally shown to survive in meat stored at temperatures below 0°C for 7 days (Hill et al., 2006), and at +58°C for only 9.5 minutes (Dubey et al., 1990), as well as at pressures below 300–400 Mega Pascal (Lindsay et al., 2006). Chemical processing of pork in 2% sodium chloride and 1.4% potassium or sodium lactate inactivates parasites within 8 hours (Hill et al., 2006) by reducing the quantity of free water (water activity index – aw). Thus, inappropriate and insufficient use of these methods allows the parasite to stay infective in meat products, or even after preparation of pork for direct consumption.

A higher prevalence of *T. gondii* infection were recorded in pigs from organic and outdoor farming systems, while new sanitary conditions at intensive breeding farms have consistently resulted in a decrease in parasitic infection (van der Giessen et al., 2007). In pigs, *T. gondii* infection usually goes unnoticed, but can sometimes cause clinical signs such as fever, lymphadenopathy, lack of appetite (and weight loss), miscarriage, stillbirth and foetal malformation, which also lead to economic losses (Li et al., 2010).

Although in France almost 23 million (22,933,667) pigs are slaughtered every year (Association BD PORC, 2013), and pork, at 34.7%, represents a major meat consumed in France (AFSSA, 2005), no nation-wide surveillance for *T. gondii* in pigs has yet been conducted. One explanation is that pork is usually eaten well-cooked or roasted, but with the influence of different cultures and new culinary habits, the choice is left to the consumer regarding how long and how intensively the meat will be cooked. Therefore, in 2013, following the objectives and methods of previous nation-wide *T. gondii* surveillances in sheep (Halos et al., 2010) and beef (data not published), a large-scale national cross-sectional survey of pigs born, raised and slaughtered in France was undertaken in order to assess the risk of *T. gondii* infection in pork, and its transmission to humans. The aim of this study was to investigate the prevalence of the parasite in pig meat from animals born and raised under two different breeding systems (intensive and outdoor pig farms), to analyse the geographical, age-related and breeding-system variations of this prevalence, as well as to isolate *T. gondii* strains present amongst pigs.

1.3. MATERIALS AND METHODS

1.3.1. Sampling strategy

In the Netherlands in 2007 different pig farming types were studied independently and the prevalence of 1% on intensive and 5% on outdoor farms was showed (van der Giessen et al. 2007). Based on the breeding practices reported in the Netherlands and France, the estimation was that *T. gondii* prevalence in France could correspond to that of the Netherlands. According to the annual pork production report (Association BD PORC, 2012) the number of samples originating from intensive farms was set at 1,500, with 300 samples coming from outdoor farms. A stratified sampling strategy was devised. Based on the national report of pork production in France for 2012 (Association BD PORC, 2012), eight regions were chosen (Fig 1), representing 92% of the annual pork production in intensive (6 regions) and in outdoor farms (7 regions). In order to obtain the same precision of prevalence estimates on a national level, the same number of samples was collected from each region; 215 from intensive and 43 from outdoor farms, respectively. Next, two levels of stratification were formed: two production types and three age categories. The three age categories were defined as piglets (up to 25kg or 2 months of age), fattening pigs (100-110kg or 8 months of age) and older, breeding pigs-sows. From intensive farms, 115 fattening pigs, 50 piglets and 50 sow samples were collected per region. From outdoor farms, 23 fattening pigs, 10 piglets and 10 sow samples were obtained from each region.

From all eight regions included in the study, 26 slaughterhouses were chosen in relation to the annual number of animals slaughtered. From each abattoir, samples were collected randomly, at least on two different occasions, in order to avoid overrepresentation of samples from the same farm.

1.3.2. Sampling protocol

Heart samples were collected, as described by Villena et al. (2012). All samples were collected from February to December 2013. In agreement with the Ministry of Agriculture (DGAL office - The Directorate for food), all selected slaughterhouses were informed about mandatory participation in this survey. In order to avoid bias and collection errors, the slaughtering dynamics of each abattoir were analysed during the 2012 year. Based on these results, exact dates (periods of intensive production), numbers of samples, and animal categories were determined for sampling in each slaughterhouse.

On the slaughter line, the abattoir veterinarians collected heart samples (minimum 200g) in sterile plastic containers labelled with unique bar codes. Samples were stored at +4°C until the arrival of the transport vehicle, and were then taken (in cooled boxes at +4°C within 12 hours of slaughtering) to the laboratories of the Institute for Animal Health at Maisons-Alfort or the National Reference Centre for toxoplasmosis in Reims. Once in the laboratory, the hearts were stored at +4°C and cardiac fluids were collected for serology analysis (Forbes et al., 2012). Within the following 24 hours, heart digestion was performed on all serology positive animals, and randomly chosen negative ones.

1.3.3. Serology test

Detection of *T. gondii* antibodies was performed on cardiac fluids by the modified agglutination test (MAT), as described by Villena et al (2012). This is a species-independent serological test, considered to be the gold standard for the detection of *T. gondii* antibodies in animals and meat (Klun et al., 2006). The antigen was provided by the National Reference Centre for toxoplasmosis in Reims, France. The starting dilution was 1:6, in accordance with a previous study in sheep (Halos et al., 2010). Six further two-fold dilutions were made, up to 1:200. All samples reactive at $\geq 1:6$ were considered positive.

1.3.4. Mouse bioassay and qPCR

Hearts were digested using trypsin, as described by Dubey et al (1998) and modified by Halos et al. (2010). Briefly, each whole heart was cut and ground slightly. Two-hundred grams were measured and incubated with trypsin at 37°C for 90 minutes (final concentration 0.25%). The suspension was then filtered, centrifuged, and the pellet was washed twice in saline solution. From the total quantity of the pellet, 300 μ l were used for DNA extraction and 2 ml were suspended in antibiotics for intra

peritoneal inoculation into two Swiss-Webster mice (CD-1) in bioassay (1 ml per mouse. Ethics committee licence no: 21/01/13-2). Mice were bled after 6 weeks from the retro orbital sinus and tested by MAT for *T. gondii* antibodies. All seropositive mice were euthanized and brains were collected aseptically. Samples of whole brain homogenates were microscopically examined for *T. gondii* cysts. Of the brain homogenates, 300 µl were used for DNA extraction. All DNA extractions were done using QIAmp mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Quantitative Polymerase Chain Reaction (qPCR), targeting the 529 bp, repetitive element (gene bank accession number AF146527) was performed as previously described by Vujanic et al., (2011).

1.3.5. Genotyping of *T. gondii* isolates

All parasite isolates were genotyped by the PCR- Restriction Fragment Length Polymorphism (PCR-RLFP) method, using 12 genetic markers including SAG1, 5'- and 3'-SAG2, altSAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico (Su et al., 2010).

1.3.6. Definition of cases

A sample that was tissue-fluid positive in MAT (dilution $\geq 1:6$), and/or from which bio-assays live parasites/parasitic DNA were isolated, was considered as positive.

1.3.7. Statistical analysis

All statistics were performed using the R Core Team (2013). R is a language and environment for statistical computing made by R Foundation for Statistical Computing, Vienna, Austria (<http://www.R-project.org/>). For the calculation of national-level seroprevalence, the number of animals slaughtered in France in 2013, for each analysed category (Association BD PORC, 2013), was used to adjust the region- and category-specific rates. Prevalence according to the age, breeding system, and region of slaughter (CATEG: ordinal scale) were analysed by generalised linear model (logistic link) (R. 3.0.3 MASS and lme4 package). Breeding system was treated as a qualitative variable (intensive or outdoor farm, reference intensive farm), as well as age (piglet, fattening pig or sow, reference: fattening pig) (Table 7). For the categorical variable - region of slaughtering (8 modalities) - the reference was the first region from the list (Aquitaine – Table 7.) The correlation between age and prevalence enabled the determination of the age category in which animals are more frequently infected, and relationship prevalence – the breeding system enabled the identification of the farming method that posed a higher risk of infection. Variables associated with positivity at $P \leq 0.1$ at the 95% confidence level were tested for co-linearity and included in a multiple logistic regression

model. Overall fit of the logistic regression model was assessed by the Hosmer-Lemeshow goodness-of-fit statistics. To calculate concordance, taking into account concordance by chance (between serology and bioassay, serology and qPCR and bioassay and qPCR), Kohen's kappa test with 95% confidence interval was used (R package fmsb).

1.4. RESULTS

T. gondii seroprevalence and strain presence in pork produced in France was analysed in eight out of 22 administrative regions based on the annual numbers of slaughtered pigs from intensive and outdoor farms.

1.4.1. Collected samples

The study was conducted on a total of 1,549 pigs (Table 7), from 752 farms. Of the 1,549, 1,342 (86.7%) were intensively bred animals, and 207 (13.3%) were outdoor pigs, which is proportional to the number of animals slaughtered from each rearing system in France. According to age, samples were collected from 133 (8.6%) piglets, 1,158 (74.7%) fattening (market weight) pigs, and 258 (16.7%) sows (Table 7), which matches with the number of pigs slaughtered in each of these age categories in the country. The number of samples from individual farms varied between one and 25 animals, and depended on the production type, age category and size of the originating farm. Since the study was based on the numbers of animals slaughtered per region, the seroprevalence results were further analysed according to the region of slaughtering.

1.4.2. Seroprevalence analysis

Overall, adjusted seroprevalence among fattening pigs from both indoor and outdoor breeding systems was 2.9% (95% confidence interval CI: 0.9-5.0%) (Fig 2).

1.4.3. Seroprevalence in pigs from intensive farms

The adjusted seroprevalence of *T. gondii* infection in pigs raised in intensive farm systems was 2.7% (95% CI: 0.0-7.0%). The highest seroprevalence was in the Bretagne region (6.3%, 95% CI: 3.3-9.3%) and the lowest in Basse – Normandie (1.6%, 95% CI: 1.0-5.0%) (Fig 2). The highest adjusted seroprevalence was found in sows (13.4%, 95% CI: 6.8-19.9%), followed by fattening pigs (2.9%, 95% CI: 0.8-4.9%), while it was the lowest in piglets (2.6%, 95% CI: 0.0-6.9%) (Table 7; Fig 2).

1.4.4. Seroprevalence in pigs from outdoor farms

Due to the small number of piglet and sow samples (five and seven, respectively) collected from outdoor farms, the seroprevalence was calculated only for fattening pigs. The adjusted seroprevalence was 6.3% (95% CI: 2.7-9.9%), the highest in Midi-Pyrénées (17.2% 95% CI: 5-33%) and the lowest in Poitou-Charentes (2.6%, 95% CI: 0.1-14%); in Bretagne, only three samples were tested and all were negative (Fig 2).

1.4.5. Live parasite isolation

Out of all 1,549 samples, parasite isolation was attempted on 160 samples (69 positive samples and 91 randomly selected negatives). In total, 41 parasite strains were isolated (Table 1), of which only one originated from an indoor fattening pig that was seronegative. All 41 strains belonged to type II, as in most European countries. The region with the highest number of the *T. gondii* isolates (10) was Midi-Pyrenees and only one strain originated from Poitou – Charentes (Table 7).

Bio-assays were performed on 11 piglet samples, but no strain was identified. Various *T. gondii* isolates were found in 27 out of 98 fattening pigs (27.55%) and 14 out of 51 sows (27.45%). From the 27 strains found in fattening pigs, 12 originated from intensive and 15 from outdoor farms. The majority of strains isolated from sows, 13 of them, originated from intensively bred animals (but 49 samples were tested), and only one came from an outdoor animal (only 2 samples were tested) (Table 7).

When adjusted according to the total number of animals in each age category, the strain prevalence in intensive fattening pigs was 0.9% (95% CI: 0.0-1.9%) and 8.8% in sows (95% CI: 3.2-14.3%). In outdoor fattening animals the overall strain presence was 10.2% (95% CI: 0.0-22.1%).

1.4.6. Concordance between serology and parasite isolation

Out of 160 bio-assays, 41 strains were found. Substantial concordance between serology and the presence of infective parasites was calculated (Kohen's kappa 0.66, 95%CI: 0.52-0.80). All digested hearts were tested in bio-assay and examined for *T. gondii* specific DNA by qPCR. Thirty-four out of 160 found positive (between 20th and 35th cycle), indicating moderate concordance between serology and PCR (Kohen's kappa 0.58, 95%CI: 0.43-0.72). Moderate accord was also obtained between two direct detection methods: bioassay and PCR (Kohens's kappa 0.58, 95%CI: 0.43-0.72).

1.4.7. Risk-factor analysis

Risk-factor analysis was performed for all 1,549 animals. Univariate analysis shows that age and breeding methods both correlated with *T. gondii* seropositivity (Table 8). The region of slaughtering was not associated with seropositivity, which excluded possibility that certain slaughterhouses collect animals from more contaminated farms.

The final multiple model included age and breeding, with a 95% (0.968) rate of correct prediction in Hosmer-Lemeshow goodness-of-fit test. The results in Table 8 display a risk of infection that is almost four times (3.62) greater for pigs raised in outdoor farms than those reared in intensive farming ($P=0.004$, 97.5%CI: 1.94-6.59). Concerning the age of the animals, compared to fattening pigs, sows were at an almost five-fold (4.63) higher risk of infection ($P<0.001$, 97.5%CI: 2.65-8.09), and there was no statistically significant difference between fattening animals and piglets ($P=0.96$)(Table 8).

1.5. DISCUSSION

There is numerous data on the seroprevalence of *T. gondii* infection in pigs throughout the world, with values ranging from 0.9% in sows from Austria (Edelhofer, 1994) to 0% in pork from the Netherlands (Kijlstra et al., 2004), to as high as 45.3% in organic farms from Veracruz, Mexico (Alvarado-Esquivel et al., 2014) and 37.8% in Argentina (Venturini et al., 2004) (Table 9). The live parasites are isolated from pigs in all parts of the world with types II and III predominant in Europe and North America (Mondragon et al., 1998; Klun et al., 2011; Dubey et al., 2012; Turcekova et al., 2013), and atypical strains more characteristic for South America and Asia (Zakimi et al., 2006; Belfort-Neto et al., 2007; Zhou et al., 2010b; Bezerra et al., 2012).

One of the benefits of intensive pig farming is that sanitary and technical control measures are at higher levels. Keeping the animals in containment does little for their natural welfare but provides higher sanitary and animal health standards. Findings of parasitic, bacterial and virus species in these conditions are proof that these environments still have not met satisfactory levels of containment. At the same time, although organic and backdoor farming provide greater animal welfare, they represent constant reservoirs of different diseases for pigs and a greater threat to public health.

This is the first nation-wide study in France that uses pork production from both systems (indoor and outdoor) and consumption data to construct the sampling strategy. The results of this cross-sectional survey of *T. gondii* infection in pigs from France show adjusted prevalence values of 3.0% at intensive and 6.3% at outdoor farms. Studies in Spain and Portugal, using the same test (MAT), showed a prevalence of 16.6% and 9.8% respectively (Garcia-Bocanegra et al., 2010; Lopes et al., 2013) while in Switzerland, using an Enzyme-linked immunosorbent assay (ELISA), Berger-Schoch

et al. (2011) showed prevalence of 23% (Table 9). The highest prevalence in Europe was recently reported in the Czech Republic - 36% among intensive breeding pigs (Bartova & Sedlak, 2011), and Romania - 30.5% in outdoor animals (Pastiu et al., 2013).

In risk-factor analysis we found that age and breeding type associated with *T. gondii* positivity. Age is often considered a major risk factor for *T. gondii* infection in pigs, as older animals have a greater chance of acquiring an infection in a longer time frame (Villari et al., 2009; Halos et al., 2010; Klun et al., 2011; Halova et al., 2013; de Sousa et al., 2014). Current study also indicates that younger age categories (piglets and fattening pigs) are more protected than breeding animals. Piglets from intensive farms are slaughtered very young (up to 45-60 days of life -25kg), a period when maternal antibodies still can be identified (Garcia-Bocanegra et al., 2010). Infected piglets cannot be detected by current methods because animals are either in prepatent period or the parasitic infection is stopped by maternal antibodies. Moreover, in our study, out of 11 piglets from which parasitic isolation was tried, no strains were isolated; from 98 fattening pigs in 27.55% and from 51 sows in 27.45%, isolation was successful. Many piglet-producing farms have outside pens for the young, but not for sows, making these facilities more vulnerable to cat and rodent access (AFSSA, 2005) and increasing the chance of infection through contact with oocysts or infected mice and rats. Therefore, piglets may import infective oocysts that during the mother's grooming can infect the sow, or cause infection in late weaning and fattening period, after maturation of the digestive system (one oocyst is sufficient to produce infection in pigs (Dubey et al., 1996)).

High prevalence among outdoor pigs is common, if we take into account that outdoor pigs are more exposed to environmental contamination (cat faeces, rodents and contaminated nutrients), as well as potential contaminants of indoor farms. In Europe the prevalence ranges from 5.6% in the Netherlands (van der Giessen et al., 2007), 17.6% in Latvia (Deksne & Kirjusina, 2013), and 23% in Switzerland (Frey et al., 2012)(Table 9). On other continents prevalence rises, reaching 27% in South China (Zhou et al., 2010a), 37.8% in Argentina (Venturini et al., 2004), 45.3% in Mexico (Alvarado-Esquivel et al., 2014), and the northern USA where Dubey et al found 90% prevalence on two organic farms (33 animals) (2012). Only Berger-Schoch et al. in Switzerland showed no statistically significant difference between indoor and outdoor breeding systems (Berger-Schoch et al., 2011). This study once again showed an increased risk of infection in outdoor pigs which may also be explained by the greater possibility of environmental contamination by *T. gondii* oocysts (Jiang et al., 2008; Feitosa et al., 2014) or, if there are no cats, higher incidence of pigs having contact with rodents, especially if rodent-control measures are absent (Garcia-Bocanegra et al., 2010; Piassa et al., 2010).

Between serology results and parasite isolation by bioassay, concordance was substantial (0.66), which suggests that the heart can be recommended as the sample of choice for epidemiological studies (Dubey, 1988; Halos et al., 2010; Wang et al., 2012). Worryingly, one strain has been isolated from a

seronegative animal. Similar results were reported from Portugal, Ireland and Slovakia (Halova et al., 2013; Turcekova et al., 2013; Esteves et al., 2014) and even the USA (Lehmann et al., 2003; Dubey et al., 2012). At the opposite, 13 positive qPCRs were obtained from seronegative animals, but without strain isolation. This can be interpreted as a lack of sensitivity of available detection methods. Considering the fast fattening period, the time between pig infection and the development of an immunological reaction could be quite limited and undetectable using existing methods. Thus, new diagnostic tools are needed in order to detect early infections in pigs and allow for more accurate risk-assessment studies. RFLP genotyping showed that type II strains are 100% prevalent among pigs in France, which corresponds with the majority of strains found in sheep (Halos et al., 2010) and bovines slaughtered in France (unpublished data). Type II was also the most prevalent in other European countries: pigs from Portugal and Slovakia (de Sousa et al., 2006; Turcekova et al., 2013), sheep from Switzerland and Serbia (Frey et al., 2012; Markovic et al., 2014) but also cats from Germany (Schares et al., 2008).

In summary, the described prevalence of *T. gondii* infection in pigs from France provides further evidence of the extensive infection of this parasite regardless of breeding systems, and thus innovative prevention measures are needed to lower the parasite burden in pigs, and consequently the zoonotic risk. Although more desirable from a market point of view, outdoor pigs are still more exposed to environmental contamination. Still, parasite isolation from pigs originating from closed, intensive farms goes to show that zootechnical containment measures are not sufficient in either of these conditions to allow the labelling of Toxoplasma-free pork on the market. Until more detailed risk-factor analysis can be performed, recommendations to farmers may include the introduction of more strict regulations at animal facilities.

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Figure 1 French pork production in numbers of animals slaughtered from A-intensive, B- outdoor pig farms, and C-regions chosen for sampling

Shades of blue (intensive pig farms) and green (outdoor pig farms) represent scale of regional pork production in France. Eight regions, coloured on map C in blue, green and red, were selected for study rendering 92% of all animals slaughtered in 2012.

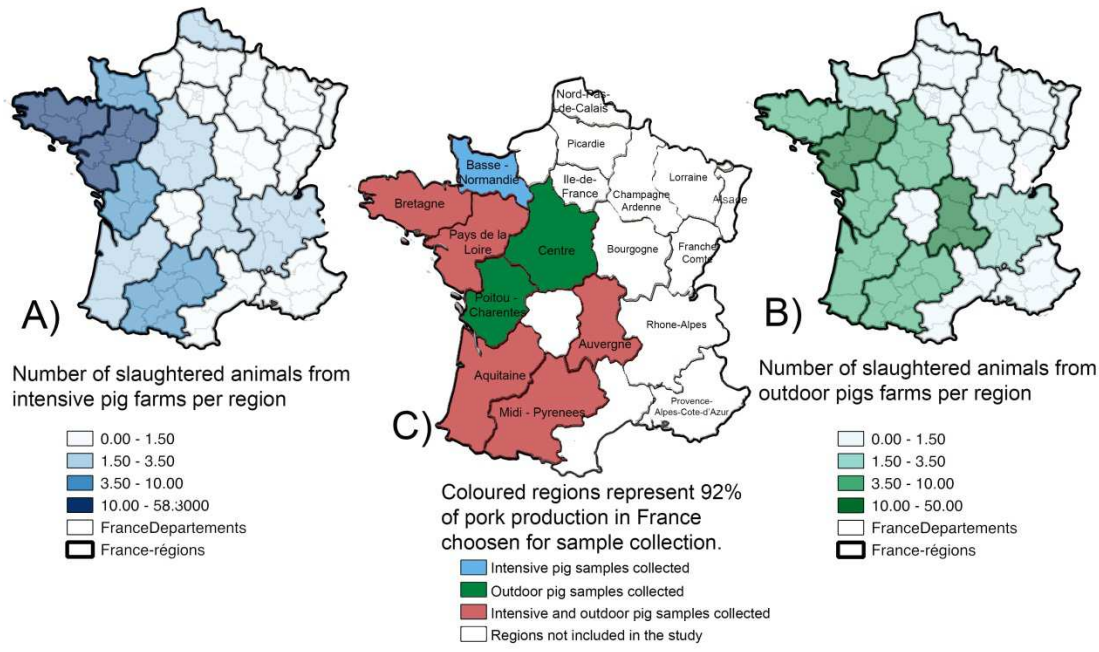
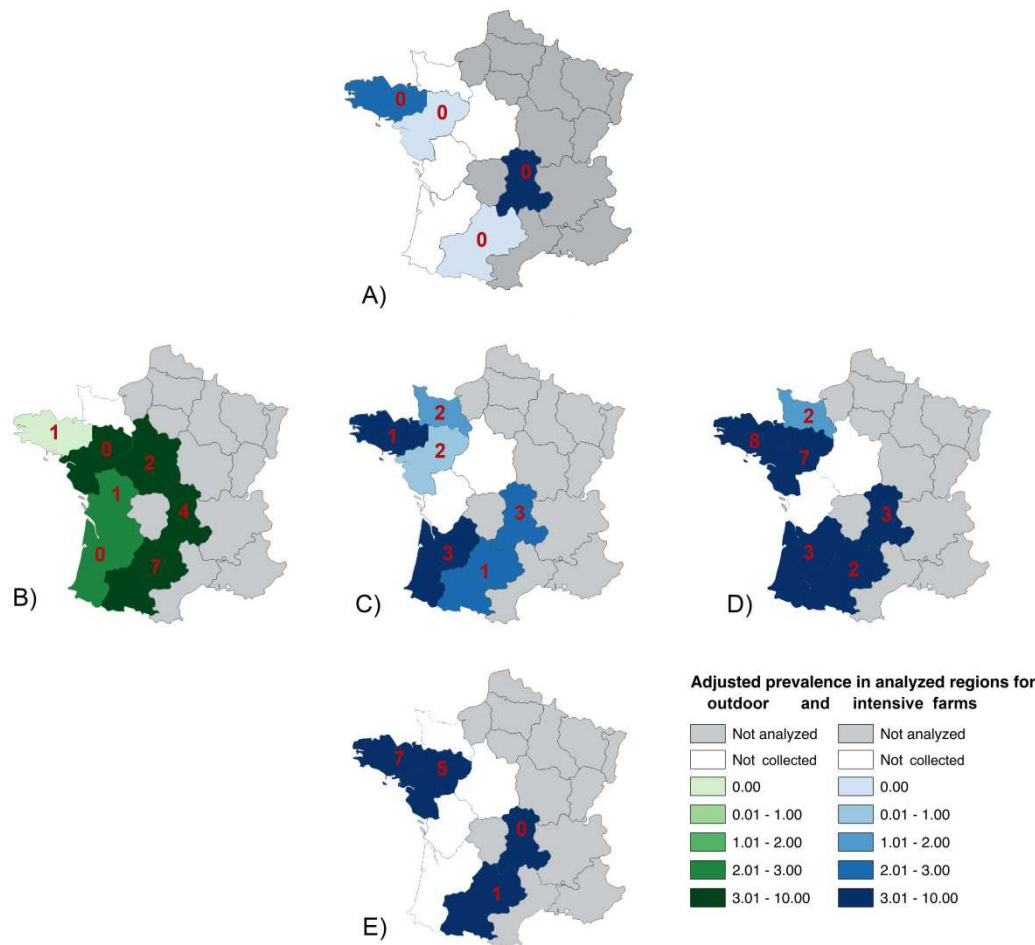


Figure 2 *Toxoplasma gondii* adjusted seroprevalence and strain isolations according to the production and age categories in analysed regions

Adjusted Seroprevalence (calculated in percentage) is presented according to: 1. Production categories: outdoor pig farming– B (green scale) and intensive pig farming – A, C, D, E (blue scale); 2. Age categories: A – piglets, B and C - fattening pigs, E - sows. Numbers of strains isolated from each category per region are written in red. For intensively bred pigs the sum of all age classes is calculated on map D. Comparison was made only between B and C where there is a noticeably higher prevalence in pigs from outdoor than indoor farms. The highest number of strains originates from fattening pigs bred in outdoor farms.



397 **Table 7 *Toxoplasma gondii* seroprevalence and strain isolation from pigs in France**

398 All collected samples are arranged per production type, age and region of slaughtering. In every sub-category the number of positive, total number of samples
 399 and prevalence are calculated. According to age sub-classes, calculations were possible only for fattening pigs. Finally, for all sub-categories of production
 400 and age, seroprevalence was adjusted according to total number of animals slaughtered in France, from each sub-class. No strains were isolated from piglets,
 401 27 from fattening pigs and 14 from sows. All strains were genotyped by Restriction fragment length polymorphism (RFLP) method as type II.
 402

Areas of 92% production of pork in France	Total number of samples collected	Sample size – positives/total (Prevalence in %)						Parasite isolation								
		Intensive farms			Outdoor farms			Total number of isolated strains	No. of parasite strains isolated from Intensive farms			No. of parasite strains isolated from outdoor farms			Genotype	
		Piglets	Fattening pigs	Sows	Piglets	Fattening pigs	Sows		Piglets	Fattening pigs	Sows	Piglets	Fattening pigs	Sows		
Aquitaine	258	0	7/222 (3.2)	0	0	1/36 (2.8)	0	3	0	3	0	0	0	0	0	II
Auvergne	245	3/40 (7.5)	3/148 (2.0)	1/25 (4.0)	0	5/32 (15.6)	0	7	0	3	0	0	4	0	0	II
Basse-Normandie	182	0	3/182 (1.6)	0	NA	NA	NA	2	0	2	0	NA	NA	NA	NA	II
Bretagne	263	1/35 (2.9)	5/150 (3.3)	10/70 (14.3)	0/5 (NA)	0/3 (NA)	0	9	0	1	7	0	1	0	0	II
Centre	43	NA	NA	NA	0	3/43 (7.0)	0	2	NA	NA	NA	0	2	0	0	II
Midi-Pyrénées	257	0/3 (NA)	3/137 (2.2)	4/86 (4.7)	0	5/29 (17.2)	2/2 (1.0)	10	0	1	1	0	7	1	0	II
Pays de la Loire	258	0/50 (0.0)	1/124 (0.8)	10/70 (14.3)	0	1/14 (7.1)	0	7	0	2	5	0	0	0	0	II
Poitou-Charentes	43	NA	NA	NA	0	1/38 (2.6)	0/5 (0.0)	1	NA	NA	NA	0	1	0	0	II
Total (positive/total)		4/128	22/963	25/251	0/5	16/195	2/7	41	0	12	13	0	15	1	0	II
Adjusted prevalence (95% CI)	1549	2.6 (0.0-6.9)	2.9 (0.8-4.9)	13.4 (6.8-19.9)	NA	6.3 (2.7-9.9) ₆₀	NA									

403

404 **Table 8 Analysis of risk factors associated with *Toxoplasma gondii* contamination of pork**

405 Univariate models show that age ($p<0.001$) and production ($p=0.004$) but not the region of slaughtering ($p=0.259$), have influence on seroprevalence of *T.*
 406 *gondii* specific antibodies in pork. In multiple analyses, all three variables were tested and the final model showed that pigs from outdoor farms are eight times
 407 more likely to be infected with *T. gondii* than animals raised in intensive farms. Sows are almost five times more likely to be parasite carriers than fattening
 408 animals, and there is no statistical difference between fattening pigs and piglets ($p=0.96$). Region of slaughtering has no influence on the prevalence of *T.*
 409 *gondii* infection in pigs ($p=0.319$).

Variable	Univariate analysis						Multiple model ⁴¹⁰		
	n	Prevalence (%)	95% CI	OR	95% CI	P- value	OR	97.5% CI	P-value ⁴¹¹
Age						<0.001			
Fattening pigs	1158	3.3	0.02-0.05	1			1		
Piglets (<25kg)	133	3	0.01-0.08	0.914	0.271-2.320		1.19	0.35-3.09	0.96
Sows	258	10.5	0.07-0.15	3.445	2.044-5.733		4.63	2.65-8.09	<0.001
Production type						0.004			
Intensive farms	1342	3.8	0.03-0.05	1			1		
Outdoor farms	207	8.7	0.05-0.14	2.41	1.379-4.215		3.62	1.94-6.59	0.004
Region of slaughtering						0.259			
Aquitaine	258	3.1	0.01-0.06	1					
Auvergne	245	4.9	0.03-0.09	0.74	0.091-6.103				
Basse-Normandie	182	1.6	0.01-0.05	0.46	0.059-3.650				
Bretagne	263	6.1	0.04-0.09	1.42	0.144-14.000				
Centre	43	7.0	0.02-0.20	0.37	0.047-2.845				
Midi-Pyrénées	257	5.4	0.03-0.09	0.32	0.032-3.180				
Pays de Loire	258	4.7	0.03-0.08	0.41	0.053-3.226				
Poitou-Charentes	43	2.3	0.00-0.14	0.49	0.062-3.853				

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Table 9 Review of *Toxoplasma gondii* seroprevalence in pigs per country/continent according to pig production systems

The table shows the number of animals included in each study serological tests used and obtained seroprevalence. The highest seroprevalence was recorded in Mexico (45.3%) by Alvarado-Esquivel et al from 2014 when Modified Agglutination Test (MAT) was used for serology. In Europe, the highest seroprevalences were found in the Czech Republic and Romania, 36% (by Enzyme-linked immunosorbent assay (ELISA)) and 30.5% (by Immunofluorescence Antibody Test (IFAT)), respectively. In all countries except Switzerland it was found that *T. gondii* is more prevalent in outdoor pigs.

Source	Country	Production type	Age category	Test used	Prevalence (%)		Number of analyzed animals	
					Per category	Total	Per category	Total
Europe								
(Edelhofer, 1994) (van der Giessen et al., 2007)	Austria	NA	NA	IFAT	NA	0.9	NA	4697
	Netherlands	indoor	NA	ELISA	0.4	NA	NA	845
		outdoor	NA		5.6		NA	
(Lopes et al., 2013)	Portugal	NA	Piglets	MAT	42.9	9.8	21	254
			Fattening		5.0		180	
(Garcia-Bocanegra et al., 2010)	Spain	NA	Breeding	MAT	13.2	16.6	53	2970
			Fattening Sows		9.7		1570	
(Berger-Schoch et al., 2011; Frey et al., 2012)	Switzerland	indoor	Finishing	ELISA	14.0	23	50	270
		outdoor	Adults		36.0		120	
			NA		13.0		100	
(Bartova and Sedlak, 2011)	Czech Republic	NA	Fattening	ELISA	NA	36	NA	551
(Pastiu et al., 2013)	Romania	Indoor	Fattening	IFAT	0	30.5	660	3595
		outdoor	Sows		12.4		371	
			NA		30.5		2564	
(Deksne and Kirjusina, 2013)	Latvia	NA	NA	ELISA	NA	17.6	NA	803

(Klun et al., 2011)	Serbia	NA*	Fattening Sows	MAT	8.3 30.0	9.2	468 20	488
(Turcekova et al., 2013)	Slovak Republic	NA	Finishing Sows	ELISA	2.1 4.3	2.2	923 47	970
(Veronesi et al., 2011)	Italy	Indoor	NA	IFAT	NA	16.4	NA	960
(Sroka et al., 2010)	Poland	NA	NA	DA	NA	17.9	NA	67
North and South America								
(Alvarado-Esquivel et al., 2014)	Mexico	Outdoor	Fattening	MAT	NA	45.3	NA	402
(Venturini et al., 2004)	Argentina	Indoor	Piglets		1		103	
			Fattening Sows		0		69	
		Outdoor	Piglets	MAT	2.7 0.4	37.8	184 30	230
			Fattening Sows		13.8 100		29 23	
(Dubey et al., 2012)	USA**	Outdoor	NA	MAT	NA	90.0	NA	33
(Samico Fernandes et al., 2012)	Brazil	NA	Fattening	IFAT	NA	12.5	NA	305
Asia								
(Zhou et al., 2010)	China	NA	Piglets Fattening	ELISA	14.9 25.2	27.0	114 671	1022
(Wu et al., 2012)	Tibet	Outdoor	Breeding Piglets		38 21.1		237 71	
			Fattening Sows	MAT	23.6 0	22.7	347 9	427
(Chikweto et al., 2011)	Caribbean islands	NA	NA	MAT	NA	23.1	NA	247

NA=Not Analysed

NA*= Production was analysed as a risk factor but the exact number of animals per production and age category cannot be determined

USA**= Data from two farms are published.

2. Section 2:

Paper submitted to Parasites & Vectors journal

Factors associated with *Toxoplasma gondii* infection in confined farrow-to-finish pig herds in Western France: an exploratory study in 60 herds

DJOKIC V.^{1,4}, FABLET C.², BLAGA R.¹, ROSE N.², PERRET C.³, DJURKOVIC -DJAKOVIC O.⁴,
BOIREAU P.³, and DURAND B.⁵

First three authors contributed equally to this study.

1. Ecole Nationale Vétérinaire d'Alfort, ANSES, INRA, Université Paris-Est, Laboratoire de santé animale de Maisons-Alfort, UMR BIPAR, Maisons-Alfort, France;
2. ANSES, Unité Epidémiologie et Bien-Etre du Porc; Université Européenne de Bretagne, Laboratoire de Ploufragan-Plouzané, Ploufragan, France.
3. ANSES, Ecole Nationale Vétérinaire d'Alfort, INRA, Université Paris-Est, Laboratoire de santé animale de Maisons-Alfort, UMR BIPAR, Maisons-Alfort, France
4. University of Belgrade, Institute for Medical Research, National Reference Laboratory for Toxoplasmosis, Dr. Subotića 4, P.O. Box 102, Belgrade 11129, Serbia
5. ANSES, Université Paris-Est, Laboratoire de santé animale de Maisons-Alfort, Epidemiology unit, Maisons-Alfort, France; Electronic address : benoit.durand@anses.fr

2.1. Summary

Background

Infection by *Toxoplasma gondii* postnatally can occur after ingestion of contaminated meat or water (tissue cysts/oocysts). In Europe, percentage of meat borne infections is estimated between 30% and 63%, out of which pork makes the most important source. The aim of this study was to investigate i) the seroprevalence of *T. gondii* in intensive pig farms from western-France; ii) the risk factors associated with seropositivity.

Methods

Stratified data and sera collection was done from November 2006 to February 2008 on 60 intensive farms from 3595 suckling-, weaned- piglets and fattening pigs. Information about three classes of risk factors were obtained through questionnaire: i) breeding characteristics, ii) farm management, iii) husbandry and hygiene. The modified agglutination test (MAT) was used for detection of anti *T. gondii* antibodies in pig sera, starting from 1/6 dilution.

Results

The individual-level seroprevalence was 6.9%, but the proportion of herds with at least one positive pig was 100%. The average within farm prevalence was 7.0%. Multivariate mixed logistic model showed an increased seropositivity risk in weaned compared to suckling piglets, and a decreasing prevalence in mid-size and large farms. Separated facilities with a Danish entry system, that clearly separates clean and dirty areas, had a protective effect on *T. gondii* seroprevalence as well.

Conclusions:

The observed *T. gondii* seroprevalence provides further evidence that even in confined conditions of pig breeding, infection occurs, and is widespread. The highest risk of acquiring *T. gondii* is at the end of weaning period. Smaller farms demonstrate higher *T. gondii* seroprevalence in confined conditions. This study also shows that Danish entry systems on farm buildings provide effective protection against *T. gondii*.

Key words:

Toxoplasma gondii, intensive pig farm, risk factors

2.2. Background

Toxoplasma gondii is one of the world's most widespread food borne pathogens. The parasite from phylum *Apicomplexa* is adapted for infection of all warm-blooded animals including humans. The characteristic of *T. gondii* is that parasite can be transmitted directly among intermediate hosts, including pigs, by predation or scavenging without the need for sexual part of the life cycle. The ingestion of food or water contaminated with different parasitic life stages (oocysts/tissue cysts) are the two major routes of postnatal transmission of *T. gondii* (16).

Infection in humans is generally benign, but may have serious consequences in the developing foetus, if maternal infection occurs in pregnancy and in immunosuppressed individuals, causing the disease – toxoplasmosis (4). In most people infection is characterized by mild, flu-like symptoms, but in vulnerable categories serious infections of foetus, nervous system and eyes can occur with even life-threatening consequences. Due to its zoonotic character, this disease represents an important public health hazard. The percentage of meat borne infections of the total number of toxoplasmosis patients has been estimated between 30% and 63% in Europe (2).

Along with sheep, pigs are the species mostly associated with human *T. gondii* infection (5). Nowadays three pig farming systems are recognized: i) intensive, ii) free-range (outdoor) and iii) organic farming. In intensive farms animals are kept in strictly confined conditions, without any access to outside environment. Pigs are separated in categories, defined as batches (contemporary group of pigs), from where all animals are transported in the same time (all in all out). In contrast, both organic and outdoor pigs have access to natural light, weather conditions and contact with other animal species. Mainly, organic pigs are bred in large open space pens without closed facilities, fed by seasonal food without chemical processing, and the system all-in, all-out is not practiced. Results from studies conducted in Europe indicate that pigs can be infected by *T. gondii* in various intensities depending upon farming system, animal age, presence of cats on site, absence of rodent control and other breeding practices (Figure 1).

Pigs destined for human consumption, have not yet been screened on a regular basis for *T. gondii* infection at slaughter in any country (10). Therefore endemic areas, the sources of pig infection, the frequency of infected pigs on the markets, and the role these animals play in human *T. gondii* epidemiology remain unknown. Comparing the prevalence between wildlife and domestic animals, no significant difference in *T. gondii* infection rates of wild boar as a reservoir, and organically/free-range bred pigs can be observed (Figure 3). These animals get infected mainly from an oocyst-contaminated environment by rooting, or by consuming infected rodents. As a consequence, the pigs not exposed to these two sources-from intensive farms, could be *Toxoplasma* free.

A nation-wide study of *T. gondii* in pigs slaughtered for human consumption in France described similar *T. gondii* seroprevalence on individual level in piglets and fattening pigs (2.6% and 2.9% respectively) (108). Most of the pigs originated from intensive farms, indicating low, but significant level of contamination. However, study was not designed to evaluate if contamination is widespread or limited to some herds.

Therefore, the aim of present work was to analyse the presence of *T. gondii* in intensive pig farms and investigate: i) the farm-level and within farm proportion of seropositives, and ii) factors associated with seropositivity. For this purpose enquiry was conducted on samples and data from 60 confined farrow-to-finish farms located in western France, the region where almost 60% of total country's pork production is localized.

2.3. Materials and methods

2.3.1. Database and serum bank

Sera samples and epidemiological data were obtained from a previous cross-sectional study devoted to pig respiratory diseases. Collection was done from November 2006 to February 2008 in farrow-to-finish farms from western-France as described previously (109). In France 92% of pig production is localized in west of the country (110). A total number of 7500 piglets and fattening pigs sera from 125 farms were on disposal, as well as associated individual and farm level data. The visit to each herd consisted of blood sample collection, face-to-face interview with the farmer and herd investigation by "Swine epidemiology and welfare unit - ANSES", as described in (109). Data related to general herd and neighbourhood description, biosecurity and hygienic practices, management and animal husbandry were collected with a standardized questionnaire.

2.3.2. Herd selection and risk factors

A written consent from organisations of producers was asked for use of the sera and meta-data in the *Toxoplasma gondii* seroprevalence survey and risk factors analysis. In Europe, farm prevalence varies from 4% on intensive farms in Netherlands to 85 % in Spain (87, 111). A farm was considered as *T. gondii* positive if specific antibodies were found in at least one pig. The expected farm prevalence was set at 20% and relative precision to 5%. The corresponding sample size was calculated to be 60 farms, which were randomly chosen among 125 available from the database. Assuming within-herd prevalence of 5%, the number of samples per farm necessary to detect *T. gondii* presence at a 95% confidence level was 60 animals.

The age of pigs was a single individual risk factor considered, with 4 classes: <1 month, 1-2 months, 2-3 months and >3 months. On all farms pigs are kept in completely confined conditions,

without any outside access neither for piglets or sows, separated in buildings identified as farrowing rooms (for sows and piglets from birth to one month of age), weaning (from one to two months old piglets), and fattening roomss (animals from 2-3 months of age are kept in separate pens from older pigs, but there are no distinctive differences in husbandry or hygiene between two age classes). Farm-level risk factors described i) pig housing conditions with three types of bedding (slatted floor, straw, combination of slated and concrete floor), food methods for food distribution (automatic feeders or manual), and ii) hygiene practices, with use of specialized boots and clothes, presence of a Danish entry (yes/no), and the compliance with all-in-all out management (time in days during which pens/rooms/buildings are left empty in post-weaning and fattening processes). All farms used pressurized steam for cleaning and specialized third party always performed rodent control. The number of sows (on a logarithmic scale) was used as a proxy for herd size. Finally the presence of dairy cows or beef cattle, as well as sheep, goats and poultry breeding facilities were considered.

2.3.3. Serology testing

Detection of *T. gondii* IgG antibodies was performed on sera by the modified agglutination test (MAT), as described by Halos, Thebault (47), since this is a species independent serological test. MAT is considered as gold standard for the detection of *T. gondii* antibodies in animals and meat (54, 62, 78, 112). The National Reference Centre for toxoplasmosis in Reims, France provided the antigen. Four two-fold dilutions were made starting from 1/6 to 1/50. All results were observed qualitatively (negative/positive) regardless the dilutions.

2.3.4. Statistical analysis

The overall animal as well as age class-specific seroprevalence with exact binomial confidence intervals was calculated. In positive farms, the distribution of within-farm proportion of *T. gondii* seropositive was analysed by computing the average value and the associated confidence intervals.

Because of the relatively small size of the farm sample (60 farms), risk factors for which exposure was rare (less than 5 farms) were excluded from the analysis. The univariate association between remaining individual- and farm- level risk factors was analysed using logistic models and likelihood ratio tests: risk factors for which p-value was ≤ 0.20 were selected for inclusion in the multivariate model. Absence of significant multi-collinearity was checked by verifying that variance inflation factor was < 5 , for each of the selected variables. A logistic mixed model was then used to determine the association between selected risk factors (treated as fixed effects) and the pig seropositivity, farm number being included as a random effect. Significance threshold was set to 0.05.

All statistics were performed in the R (113) (<http://www.R-project.org/>), using the MASS, lme4 and car packages (114-116).

2.4. Results

To analyse the prevalence of *T. gondii* in intensive pig farms, 60 samples of suckling and weaned piglets, as well as fattening pigs were collected per herd in western France. Analysis was done on a total of 3595 sera from 500 suckling-, 389 weaned piglets and 2532 fattening pigs.

2.4.1. Animal, farm and within-farm levels of *T. gondii* frequency of seropositivity

All herds had at least one seropositive pig, thus making the frequency of seropositive farms at 100% (95% CI: 94%-100%). When only one age category was observed, farm prevalence in suckling piglets (<1month) was 50% (95% CI: 33%-57%), as well as in weaned animals (1-2 months old - 48%, 95%CI: 27-69%), and 2-3 months old fattening pigs (48%, 95% CI: 35%-62%). However, farm prevalence was higher in pigs older than 3 months: 82% (95% CI: 70%-90%).

The overall animal-level seroprevalence was 7.0% (95% confidence interval [CI]: 6.2%-7.9%), and varied according to the age class: 5.3% in <1 month old suckling piglets (95% CI: 3.6%-7.6%), 8.5% in 1-2 months of age weaned animals (95% CI: 5.9%-11.9%), 9.5% in pigs 2-3 months old (95% CI: 7.6%-11.7%), and 6.0% in animals older than 3 months (95% CI: 4.9%-7.1%) (Table 10).

Mean within-herd seroprevalence values were very close to the individual-level prevalence, with an overall value of 7.0% (95% confidence interval [CI]: 5.3%-8.6%). This was also true when considering the age classes separately: 5.4% in <1 month animals (95% CI: 3.0%-7.7%), 7.9% in 1-2 months old animals (95% CI: 3.0%-12.8%), 9.5% in 2-3 months old animals (95% CI: 5.0%-14.0%), and 6.0% in animals >3 months of age (95% CI: 4.4%-7.5%).

2.4.2. Risk factor analysis

The variable selection procedure led to the inclusion of 5 risk factors in the multivariate mixed model: the age class, the herd size (number of sows on a logarithmic scale), the use of specialised clothes inside production units, the presence of a Danish entry, and the presence of dairy cattle (table 10).

In the multivariate mixed logistic model, a significant association between age class and seroprevalence was observed, with increasing odds-ratios for 1-2 months old weaned piglets (OR=1.8,

p=0.04,) and for 2-3 months old fattening pigs (OR=2.0, p=0.002) when compared to suckling animals (<1 month of age). However, seroprevalence in >3 months pigs was not significantly different from the one observed in <1 month piglets (p=0.5) (Table 11). Increasing herd size had a significant protective effect on seroprevalence, with an OR of 0.87 (p=0.04) for an increase from the 25% percentile (139 sows/farm) of the distribution to the 75% percentile (251 sows/farm). Dairy cattle presence on the same farm had no influence on *T. gondii* infection in pigs (p>0.05). The presence of Danish entry had a protective effect with an OR of 0.58 (p=0.04) (Table 11).

2.5. Discussion

For the last 15 years, surveys of *T. gondii* in pigs raised and consumed throughout Europe were reported from 14 countries (Figure 3). All age categories were analysed (piglets, fattening pigs, reproduction animals). In 11 studies sample collection was done at the slaughterhouse, allowing estimation of animal-level prevalence, but not farm-level and within-farm prevalence. Farm-level studies showed that the major risk factors associated with *T. gondii* infection in pigs were breeding method, animal age, presence of cats and absence of rodent control (78, 86-88, 90, 108, 117-119). Out of five studies that reported different prevalence between intensive and outdoor farming, four showed significant differences between two breeding methods, and only Berger-Schoch, Bernet (88) had contradictory results in Switzerland. Studies from Portugal, Spain, France, Serbia, Switzerland, Slovakia, and Germany showed that older age categories, from any breeding method (intensive and outdoor), have higher seroprevalence levels (79, 86, 88, 89, 108, 118-120) (Figure 3). As a source of contamination for intensively bred pigs in Europe, studies from Spain and Romania identified cats, and reports from Italy and Spain associated lack of rodent control (86, 90, 111, 121) (Figure 3). At the same time, study from the Netherlands showed significantly lower *T. gondii* seroprevalence in intensive pig farms in animals reared in strictly confined conditions (87). In present study, the piglets and fattening pigs from closed facilities in France were tested for the presence of anti- *T. gondii* antibodies.

The proportion of 100% positive farms indicates a widespread contamination of pig farms. This is confirmed by the similarity between the animal-level and the average within-farm seroprevalence: 7% in both respective cases. Indeed, this indicates that seropositive animals are not clustered in a few herds but are spread over the 60 studied farms and correspond to a limited number of animals in every herd. The farm and animal sample analysed in the present study was taken from a previous cross-sectional survey, in which farms had been included based on the history of respiratory diseases in pigs (109). For this reason, the farm and animal sample cannot be considered representative of the French population of farrow-to-finish farms, although no obvious association exists between the occurrence of

respiratory diseases and *T. gondii* circulation (other than hygienic conditions which were explicitly considered in the present study). Indeed, the animal-level seroprevalence of 7.0% reported here corresponds to those reported in Serbia, Portugal and Austria, in pigs from same type of confinement (45, 120, 122).

The multivariate model of pig seroprevalence indicated a significant association between seropositivity and age class. Duration of maternal antibodies from naturally infected sows in piglets lasts from three to six weeks of life (123) and in experimental conditions up to 120 days (124). In this period, with the decrease in the concentration of specific *T. gondii* antibodies, young animals become susceptible to infection. The earliest age piglets can get infected orally in experimental conditions is between fourth and fifth week of life (91). This goes to show that in piglets younger than one month, a *T. gondii* seropositive result may be attributed to maternal antibodies. In the same time in experimentally infected piglets, newly formed antibodies can be detected only after 2-3 weeks (91, 125). Therefore, in the 1-2 months old animals, significantly higher seroprevalence than in suckling piglets suggests that the seropositive results may in part be due to newly established infections. Furthermore, the higher odds-ratio observed for 2-3 months old animals implies that most probably infection occurs at the end of second month of life, when feeding becomes more diverse. In the cross-sectional survey of respiratory diseases on which the present study is based (126) some pig sera were analysed for infectious agents responsible for alterations of pig's immune system, such as PCV and PRRSV. These analyses aimed at documenting viral circulation at the batch level, and the individual serological results were unfortunately not available for the 3595 pig samples used for *T. gondii* analysis. For this reason, seropositivity for PCV and/or PRRSV could not be considered as risk factors of *T. gondii* infection in pigs. However, the available data showed that seropositive results for PCV and PRRSV were found mainly in animals older than three months. This could explain the lower proportion of *T. gondii* seropositive results in fattening pigs than in weaned piglets.

Only the study from Serbia showed farm size as a risk factor (120). Our study shows, as well, that small pig farms have higher risk of being infected with *T. gondii*, which may suggest a lower technical and hygienic level in small producers.

The present study shows that installation of Danish entry on the pig breeding farms decreases the *T. gondii* seropositivity risk. In the confined conditions of intensive pig farms, Danish entry is a closed facility with several doors that open successively, one at a time, upon entering the breeding facility. In the majority of cases it serves for boots and clothes exchange and sanitation, and prevents direct entering into the buildings. In these conditions even if there are other animal species (potential sources of *T. gondii* infection such as rodents, small birds and cats), their contact with pigs is less likely.

2.6. Conclusion

The described prevalence of *T. gondii* in intensively bred pigs from western-France, complements the results of a previous animal-level, nation-wide study, by demonstrating that even in confined conditions of pig breeding, parasite infection exists and that current zoo technical and hygienic measures have not met satisfactory standards for production of Toxoplasma free pork. This is the first in depth analysis of intensive farm conditions and their influence on parasite prevalence. Current study shows that maternal *T. gondii* antibodies are present in suckling piglets, and that the risk of acquiring parasite occurs at the end of weaning period. The presence of other productions on the same farm does not have any impact on *T. gondii* infection. However, small farms where pig breeding is probably not the primary activity, demonstrated increased *T. gondii* seroprevalence levels even in confined conditions. This study also shows that Danish entry on farm buildings appear to be an effective protection measure from *T. gondii* infection. Additional studies are necessary to focus on specific reservoirs of infection for intensive farms.

Figure 3 Literature review of *T. gondii* prevalence in wild boar, organically and outdoor bred pigs.

The results of *T. gondii* investigation in wild boar and pigs from organic/ outdoor farms show small differences in seroprevalence. The seroprevalence ranges from 0.5% in wild boar from island of Corsica to 63% on three organic farms in Austria. These results show those wild boars are important reservoirs for the parasite and that both organic and outdoor farming system do little to prevent infection in domestic pigs. Therefore, meat from these animals represents one of the major sources for human health as well. Used literature: intensive farming: (43, 45, 78, 79, 86-90, 108, 117-119, 121, 127, 128); outdoor/organic farming: (45, 87, 90, 108, 129, 130); wild boar: (119, 131-139) [25, 41-49]

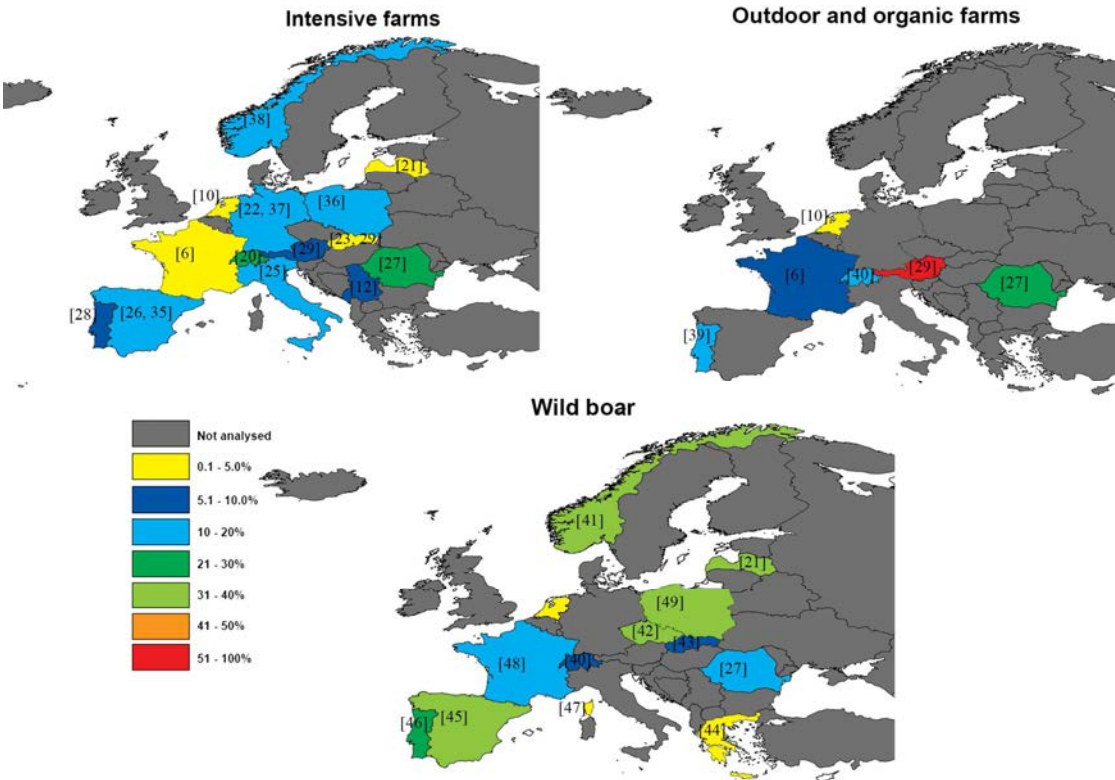


Table 10 Analysis of farm-level and individual-level risk factors responsible for *T. gondii* infection of pigs from confined farrow-to-finish productions.

Out of risk factors for which exposure is not rare (>5 farms), statistically significant influence on *T. gondii* seroprevalence in pigs have presence of dairy production, farm size, Danish entry system and individual age of animals. These factors entered final mixed logistic regression model.

				283				
Risk factor				N	Animals - positive/total (%)	<i>p</i> - value		
Farm level factors	Dairy production					<0.001		
	No					54	207/3236 (6.4%)	286
	Yes					6	44/359 (12.3%)	
	Number of sows per farm ¹					<0.001		
	0-140					15	68/885 (7.7%)	288
	141-211					16	55/970 (5.7%)	
	212-255					14	65/838 (7.8%)	289
	>256					15	63/902 (6.9%)	
	Use of separate cloths					0.187		
	No					20	92/1193 (7.7%)	
	Yes					40	159/2402 (6.6%)	
	Use of separate boot					0.937		
	No					20	95/1198 (7.9%)	
	Yes					40	156/2397 (6.5%)	
	Danish entry					<0.001		
No					43	172/2487 (6.9%)		
Yes					17	77/1048 (7.3%)		
Individu al level factors	Age in days					0.002		
	≤31					545	29/545 (5.3%)	296
	>31 and ≤62					438	36/438 (8.2%)	
	>60 and ≤100					790	76/790 (9.6%)	297
	>100					1822	110/1822 (6.04%)	298

¹Classes correspond to quartiles of the distribution, on a logarithmic scale

Table 11 Mixed logistic regression model of *T. gondii* seroprevalence in pigs from farrow-to-finish farms.

This model demonstrates that weaned piglets and 2-3 months old animals are more susceptible to be seropositive than suckling piglets (OR 1.8 and 2.0 respectively). Larger farms are more protected against *T. gondii* infection than small producers (OR 0.87, p-value=0.04). Use of Danish entries on the farm protects against *T. gondii* infection as well (OR 0.58, p-value = 0.004)

Risk factor	Value	Odds-ratio (95% CI)	p-value
Age class	<1 month – suckling piglets	1	
	1-2 months – weaned piglets	1.8 (1.0-3.2)	0.04
	2-3 months – young fattening pigs	2.0 (1.3-3.2)	0.002
	>3 months – fattening pigs and finishers	NS	>0.05
Herd size (number of sows on a logarithmic scale)	Increase from the 25% percentile of the distribution (139 sows) to the 75% percentile (251 sows)	0.87 (0.76-0.99)	0.04
Presence of dairy cattle	No	1	
	Yes	NS	>0.05
Presence of Danish entry	No	1	
	Yes	0.58 (0.34-0.99)	0.04
Use of specialised clothes inside production units	No	1	
	Yes	NS	>0.05

3. Section 3

Working title: Relationship between direct and indirect detection methods of *Toxoplasma gondii* in naturally infected pigs

3.1. Summary

In France, the concordance between serology, bioassay and PCR-based detection of *Toxoplasma gondii* in pigs was assessed on edible tissues by serology (Modified agglutination test (MAT)) on 140 selected samples. The MAT analysis was performed on cardiac juice (140), and a limited number of sera (118). In the same time, the aim of this study was to compare mouse bioassay, quantitative PCR (qPCR) on heart digest and Magnetic Capture PCR (MC-PCR) on diaphragm as direct detection methods for diagnostic of pork infection with *T. gondii* and correlate their results with MAT analysis. Out of the 140 samples, antibodies were detected in 61% of pigs according to the sera and in 43% of cardiac juice. The concordance between indirect diagnostic results was fair (0.35 95%CI: 0.18 – 0.52). When compared the MAT technique performed on two different matrices (sera and cardiac juice) with the direct detection methods (mouse bioassay, qPCR and MC-PCR), the highest kappa values were observed between MAT on cardiac fluid and mouse bioassay (0.66) or results with any direct detection method (0.41). Analysing the direct detection methods, qPCR on heart digest was the most sensitive with 69 (43.1%) positive results, followed by mouse bioassay with 41 (27.7%) and MC-PCR on diaphragm with 27 (16.9%) positive results. PCR-based direct detection techniques are in positive concordance with the finding of live parasites by bioassay in mice. The best concordance was found between qPCR and mouse bioassay (0.50), however the isolation of *T. gondii* from a seronegative pig indicate still high risk for consumers. The detection of IgG antibodies by MAT can be used to indicate the risk for consumers consuming pork. However, viable *T. gondii* was also detected in about 6% of seronegative pigs; disagreeing with the use of serological screening to classify the meat of individual animals as *T. gondii* free. Moreover, the ability to identify animals harbouring *T. gondii* using an indirect detection method is influenced by the assay and matrix used for antibody detection. There is little variation in parasite load between different skeletal muscles in pigs. Clear predilection sites (brain, heart and lung) have been identified in MC-PCR based studies described in literature. Comparing recovery rates and parasite loads in the different species, this is hampered by differences in study design and sampling.

3.2. Introduction

Apicomplexa parasite *Toxoplasma gondii* is the World wide spread food-borne pathogen. Humans and animals, postnatally acquire infection mainly by ingestion of environmentally resistant forms - oocysts, or tissue forms – cysts. Although generally mild in cases of compromised immune system, infection can have serious consequences in i) developing foetus upon maternal infection in pregnancy; and ii) in immunosuppressed individuals; causing the disease – *Toxoplasmosis* (4).

Pork is considered to be an important source of *T. gondii* infection (95, 140). In the USA pork and *T. gondii* are ranked second among top 10 pathogen-food combinations in terms of annual disease burden (141). *T. gondii* seroprevalence in pigs varies throughout the world ranging from 0% in Brazil – Minas Gerais region, Malaysia and China (142-144), to 53.4% in China– Zheijang province and 60-65.8% in Brazil – Rio de Janeiro and Belem regions (145). In Europe, the prevalence ranges between 0.3% in intensively bred pigs from Netherlands to 63.5% in animals bred on organic farms in Austria (45, 87). In South and Central America, strain presence was detected in seropositive pigs between 14.3 and 55.3% of the cases, although the seroprevalence in these studies varied from 17.1%-36% (146-149). In Europe out of 14% seropositive pigs in Switzerland PCR detected 0 positives, and in the same time DNA was found in 21 out of 21 ELISA positive animals in Slovakia (89, 150). In four European studies that used bioassay, 26.7% of seropositive intensively bred pigs from Serbia harboured parasites, while in 88.9% of outdoor animals in France *T. gondii* was isolated (108, 120). In the same time live parasites or parasitic DNA were found in raw and cured pork across the World (38, 39, 140, 151-155). These data suggest that there is a positive relationship between detection of antibodies in sera and presence of viable tissue cysts of *T. gondii* in the pork. Nevertheless, the overall parasitic burden in pork and percentage this meat makes on the world markets is unknown (10). Although ELISA and IFAT tests showed higher sensitivities and specificities for detection of specific anti - *T. gondii* antibodies in pig sera (11, 60, 112), MAT on tissue fluids was in higher concordance with isolation of infectious parasites (62, 156). In the same time, when parasitic DNA is found in the meat, real risk for human infection with *T. gondii* has not been assessed. Both mouse and cat bioassays require specialized animal facilities and strained stuff, are time consuming and expensive to perform. In order to facilitate quantitative screening of large numbers of pork samples Opsteegh, Langelaar (157) developed technique based on magnetic capture of parasitic DNA and quantitative PCR (MC-PCR). MC-PCR performed on diaphragm samples from experimentally infected pigs showed high concordance with mouse bioassay, but has not been tested on the samples of naturally infected pigs. Therefore, the aim of this study was to: i) compare detection of specific IgG anti- *T. gondii* antibodies by modified agglutination test (MAT) in pig sera and cardiac juices; ii) assess the correlation between detection of *T. gondii* antibodies by MAT in two matrices (serum and cardiac juice) and either infective parasites or parasitic DNA detection by mouse bioassay qPCR on tissue digests and magnetic capture PCR (MC-PCR) on diaphragm tissue; iii) examine concordance levels between mouse

bioassay, qPCR on heart digest and MC-PCR on diaphragm and determine the best direct detection method for diagnostic of *T. gondii* contamination in pork. For this purpose, results from previously conducted slaughterhouse study in France were used.

3.3. Materials and Methods

3.3.1. Pig samples

In 2013 nationwide study of *T. gondii* prevalence in pork from French intensive and outdoor farms was conducted (108). Briefly, 1549 pig heart samples (200g), and corresponding 1066 blood (50ml), as well as 1450 diaphragms (100g) were obtained from 26 slaughterhouses throughout 8 French regions. Out of this sample base, direct isolation of parasites or parasitic DNA was tried from hearts and diaphragms of 160 pigs.

3.3.2. Indirect detection test - MAT

Detection of *T. gondii* specific IgG antibodies was performed by the Modified Agglutination Test (MAT), as described by (42, 47), with antigen provided by the National Reference Centre for toxoplasmosis in Reims (France). Six, two-fold dilutions were made starting from 1/6 to 1/200. In the laboratory blood samples were left for 2 hours at the room temperature and then centrifuged for collection of sera. Upon collection, hearts were left at +4°C for several hours to cull, and cardiac juices – mixture of blood and tissue fluids, were separated from the transport bags. In total specific anti *T. gondii* antibodies were found in 88 pigs, either in sera or cardiac juices. From 88 MAT positive pigs, sera were missing for 16 animals, so that in total agreement between results of MAT on sera and cardiac fluids was calculated on 98 samples. Additionally, 42 cardiac juices were analysed for comparison with direct detection methods.

3.3.3. Direct detection of infectious parasites (mouse bioassay) or parasitic DNA (qPCR and MC-PCR)

Mouse bioassay and qPCR was done on 160 pig hearts, while corresponding diaphragm samples were analysed by MC-PCR. Out of 160 samples, 98 corresponded to pigs which sera and cardiac fluids were used for MAT analyses, and additional 42 (43%) MAT - cardiac juice negative animals (sera were missing) were randomly selected.

Two hundred grams of fresh pig heart were artificially digested in trypsin, as previously described (108), and final volume (3 - 5ml) was used for mouse bioassay (1ml/mouse) and DNA

extraction (300µl). Out of 160 performed bioassays 12 had to be cancelled because of the technical problems. Therefore, mouse bioassay results could be compared to other diagnostic methods for 148 pigs.

After DNA extraction using QIAmp mini kit (Qiagen GmbH, Hilden, Germany), qPCR targeting the 529bp, repetitive element (gene bank accession number AF146527) (108). For one sample qPCR was positive, but the reaction curve did not correspond to positive controls, and this sample was excluded from further analysis. That left the total of 159 qPCRs on pig heart for further analysis.

Diaphragm samples were frozen at -20°C for several months. Fat was removed from defrosted diaphragms and a 100g of muscle tissue was treated by MC-PCR method, previously described by Opsteegh, Langelaar (157).

3.3.4. Statistical analysis

To calculate concordance (qualitative agreement - positive/negative) beyond chance between the concentration of IgG antibodies determined by MAT in sera and cardiac juice Cohen's kappa test with 95% confidence intervals was used. The same analysis was performed to analyse accordance between MAT and direct detection of parasites in pig tissues (mouse bioassay, qPCR and MC-PCR). For this comparison three direct detection methods were considered separately and as a combined variable of overall direct detection positive animals. Six different concordance categories were determined as described by Landis and Koch (158): i) < 0.00: no concordance; ii) 0.00 – 0.20: poor; iii) 0.21- 0.40: fair; iv) 0.41 – 0.60: moderate; v) 0.61 – 0.80: substantial; vi) 0.81-1.00: almost perfect. MAT is a qualitative test for diagnostic of IgG antibody concentrations. Therefore, to determine the most sensitive matrix for diagnostic of parasite infection in pigs, correlation (agreement among all 7 dilutions, each observed separately) between MAT results on sera and cardiac meat juice Pearson's product moment correlation test was used with the same agreement interpretation categories as for Cohen's Kappa. Sensitivity, specificity, positive and negative prediction values and likelihood ratios of MAT on both sera and cardiac juices was calculated on first 3 dilutions (1:6, 1:12 and 1:25) with each direct detection method as reference in separate analysis. Sensitivity and specificity of MAT on two pork fluids showed precision and predictive values in comparison to mouse bioassay, qPCR and MC-PCR. In order to determine the best cut off for MAT receiver operating characteristics analysis was performed on all three matrices separately with results of bioassay as reference (pROC package).

All statistics were performed using the R (113) (<http://www.R-project.org/>). Cohen's kappa was calculated in fmsb package(159). For calculation of sensitivity, specificity, positive and negative prediction values and likelihood ratios caret package was applied (160). For ROC analysis pROC package was used

3.4. Results

3.4.1. MAT concordance and correlation between sera and cardiac juice

The results of *T. gondii* - specific IgG detection by MAT on two different matrices are summarized in Table 12. The concordance and correlation analysis has been performed pairwise for 118 samples. Fair kappa values were observed between sera and cardiac juice (0.35; 95%CI: 0.18 – 0.52) (Table 13). In the same time, by analysing qualitative values of MAT results on two matrices, almost perfect correlation was observed between sera and cardiac juice (0.8; 95%CI: 0.73 – 0.86), with more discordant results on lower dilutions (1:6, 1:12, 1:25 and 1:50) with 56 discordant out of 85 samples tested. Higher correlation is observed between MAT on cardiac juice and MAT +1 dilution (ig 1:6 and 1:12) on sera.

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

The presence of infectious *T. gondii* was demonstrated in 41 out of 148 pig hearts (27.7%), by mouse bioassay. Out of the 159 samples tested by qPCR, 69 (43.4%) had a positive amplification of *T. gondii* specific 529bp repetitive element. MC-PCR targeting the same, 529bp fragment of *T. gondii*, found parasite's DNA in 27 (16.9%) pig diaphragms. Four pigs were only bioassay positive, while parasitic DNA was demonstrated in 37 animals, with negative parasite isolation results. Five pigs were proven positive only by MC-PCR, 29 only by qPCR (Figure 4). Presence of *T. gondii* was demonstrated with two diagnostic techniques in total of 26 pigs, while 14 animals were diagnosed with infectious parasites and parasitic DNA by all three direct detection techniques (Figure 4). In total 99 samples had concordant result in mouse bioassay and qPCR with recovery rate of 0.74 from positive and 0.16 from qPCR negatives. This resulted in moderate kappa value of 0.5 (95%CI: 0.35-0.66). Kappa value between MCPCR and bioassay had fair value of 0.36 (95%CI: 0.16-0.56) with almost exact recovery rate from both MCPCR positives (0.82) and negatives (0.82).

3.4.3. Sensitivity and specificity of MAT on sera and cardiac juices

The ROC analysis was performed on MAT results of sera and cardiac juices separately using the mouse bioassay results as a reference. For both matrices ROC analysis showed ideal MAT cut off set on 1/9. The higher probability for exact results of ROC was calculated for cardiac juices (AUC: 0.89- cardiac juices, AUC: 0.814 - sera) (Figure 5). The sensitivity and specificity of MAT on cardiac fluid was set to 0.829 and 0.850, respectively while for sera was calculated Se=0.861, and Sp=0.707.

Results of MAT analyses on sera and cardiac juice were compared to direct detection methods. Since for MAT two fold dilutions, starting with 1/6 (1/6, 1/12, 1/25, 1/50, 1/100, 1/200) was made, sensitivities and specificities of MAT on both matrices were calculated for dilutions of 1/6, 1/12 and 1/25 separately. Results of mouse bioassay, qPCR and MCPCR were used as references for sensitivity and specificity calculations (Table 14). The lowest sensitivity was recorded for MAT on sera dilution 1/6 when MC-PCR was a reference (Se=0.286), while highest was calculated for MAT on cardiac juice dilution 1/25 when mouse bioassay was set as referent (Se=0.935) (Table 14). MAT on cardiac juice dilution 1/25 showed lowest specificity for qPCR results (Sp=0.517), while the highest result was calculated for sera on 1/6 and 1/12 dilutions (Sp=0.969). Overall, highest values of sensitivity and specificity were shown by MAT on cardiac juices when first two dilutions were used (1/6 and 1/12) (Se=0.848 and 0.913, Sp= 0.865 and 0.838, respectively) (Table 14). When MAT on cardiac juice is positive on dilutions of 1/6 and 1/12 the chances that bioassay on the heart of the same animal will be positive rise 6.27 and 5.63 times, respectively (Table 14). Results from Table 14 demonstrate that qPCR on heart digest can be used to predict the results of bioassay (Se=0.739 and Sp=0.838) but the other way around is not possible (Se=0.919 and Sp=0.564) (Table 14).

The best kappa value was shown between cardiac juice and mouse bioassay (Cohen's kappa= 0.66, 95%CI: 0.52-0.80) (Table 14). Results of *T. gondii* antibody detection from sera had fair correlation with bioassay (Cohen's kappa= 0.28, 95% CI: 0.10-0.45) but poor both with qPCR and MC-PCR (Cohen's kappa= 0.20 and 0.05, respectively) (Table 14). When results of any direct detection method were compared to MAT on two matrices moderate kappa agreement was established with cardiac juice (Cohen's kappa= 0.41), while sera showed the poorest concordance (Cohen's kappa= 0.18) (Table 14).

3.4.4. Concordance between direct detection methods

When compared three direct detection methods, moderate concordance was observed between mouse bioassay and qPCR (Cohen's kappa= 0.50) (Table 15), while only fair concordance could be shown between two DNA detection methods (Cohen's kappa= 0.21).

3.5. Discussion.

For testing the presence of specific anti - *T. gondii* IgG antibodies in pig sera or tissue fluids, MAT demonstrated higher sensitivity than ELISA, IFAT, LAT, or IHAT (44, 62, 156). In different ELISA analysis of pig sera mainly TgSAG1, TgSAG2, TgGRA7, TgGRA14 were used as an antigen

(60, 112, 161). In the same time, the best concordances were obtained between MAT with LAT, ELISA, and cELISA (43, 44, 156). Though all contemporary ELISA for pigs showed high sensitivities and specificities when sera were tested, their performance on tissue fluids was lower than MAT (62). In order to obtain tissue fluid from pig carcasses meat or organ samples have to be frozen at -20°C for several hours (24h) (162, 163). The intensity of more than 2000 animals slaughtered per hour in abattoirs (110), does not allow the use of tissue fluids in regular *T. gondii* diagnostics on the slaughter line. Therefore, for this study culled juices from fresh heart samples (mixture of blood and tissue fluids collected without freezing) were compared with sera for detection of anti *T. gondii* antibodies by MAT. This is the first report that compares the MAT performance on different tissue liquids of same animals as well as with direct detection of live parasites and parasitic DNA.

3.5.1. Cut off analysis for MAT on sera and cardiac juice

In routine human diagnostic of *T. gondii* infection MAT has been used as described by Desmonts and Remington (42), starting from dilutions 1:20 or 1:25 (164-166). The use of MAT 1:20 dilution in human diagnostic served as a basis for studies of *T. gondii* natural infection in animal studies as well (167-175). But seropositive animal does not always harbour infectious parasites in edible tissues (176). Hence, new studies try to determine the concordance between concentrations of specific *T. gondii* antibodies and isolation of infectious parasites in meat (47, 49, 54). The lowest dilution reported in literature – 1:6 was chosen to start the analyses in order to investigate the cut off values for sera and cardiac juices, When sheep cardiac juices are MAT positive in dilutions $\geq 1:16$ isolation of infectious *T. gondii* from heart digest was successful in 65% of cases, while bioassay failed to isolate parasites in 95% of animals when MAT was $<1:16$ (sensitivity= 0.95, specificity=0.59)(54). In this study, ROC analysis set the best cut off for both pig serum and cardiac juice at 1/9, with similar sensitivities (sera=0.86 and 0cardiac juice=0.83), and specificities,(0.71 and 0.85, respectively), as well as moderate predictive ability of the test (AUC=0.888). In pigs MAT cardiac juice positive on dilution 1:12, 79.5% of parasite isolations was successful, while from animals MAT $<1:12$, bioassay was negative in 93.3% of cases. Since MAT is a species-independent serological test, commonly used with animal matrices to detect specific anti - *T. gondii* IgG antibody presence (77), we can conclude that MAT dilution 1/6 is not specific neither for sheep nor for pigs when both sera and cardiac juices are tested. In the same time difference of one third in MAT cut-offs (1/16-sheep and 1/9-pigs) shows higher parasitic burden in pigs than sheep. Nevertheless isolation of one strain MAT negative as well as 2 strains MAT 1/6 positive on cardiac juice (in total 3/140) is acceptable error when considering sensitivity of 79.5%.

3.5.2. Comparisons between MAT on sera and cardiac juices

In 2015 Wallander, Frossling (177) compared detection of specific anti – *T. gondii* antibodies in sera and seven different meat juices of experimentally infected pigs, using the commercial ELISA. Although this study showed that meat juice is not a homogenous serological matrix for diagnostic of *T. gondii* infection, the highest correlation was calculated between heart juice and sera. However, when compared detection of antibodies in heart juice and sera by MAT with isolation of live parasites in bioassay, MAT showed higher sensitivity than ELISA (62). In our study, out of 148 pig samples, antibodies were detected by MAT in 61% of sera and in 39.1% of cardiac juices, while infectious parasites were isolated in 28.6% of bioassays. However, concordances amongst MAT results were low according to the Cohen's kappa test. Meanwhile Pearson's product moment correlation test, comparing results obtained on all seven dilutions, showed almost perfect agreement between MAT on sera and cardiac juice (Corr = 0.81; 95%CI: 0.73 – 0.86). Moreover, the existing difference between two matrices is more evident on lower dilutions (1/6, 1/12, 1/25, 1/50), where sera always showed higher concentration of antibodies. The samples for this study were collected at the slaughterhouses from naturally infected pigs of all ages. Therefore, time between infection of animals and their slaughter as well as incubation period was impossible to determine. In pigs, as in other mammals first immune response can be detected during the parasitaemia in blood (178, 179) two weeks after infection (91, 125). If the infection was recent or parasitic load was low, the immune system of certain animals would have a little or no time to develop adequate response to parasites and MAT detectable concentrations of antibodies in all tissues would not be accomplished. Therefore, the presence of anti - *T. gondii* antibodies in heart juice would depend on the blood concentration in the tissue, left after the animal was slaughtered. This means that a large proportion of the discordances might be consequence of low antibody concentrations, low homogeneity of cardiac juice compared to sera and tissue fluids, and insufficient sensitivity of MAT performed on such samples.

3.5.3. Concordance results between direct detection methods

Out of three direct detection methods, qPCR on heart digest was the most sensitive with 55 (42.6%) positive results, followed by mouse bioassay - 37 (28.7%) and MC-PCR on diaphragm - 23 (17.8%) positive results (figure 6). For *T. gondii* infection in pigs, brain is the predilection site, but in the absence of this organ, heart is ranked as second in mouse bioassay and third in MC-PCR, while muscle tissue was on fourth position (91, 180, 181). In this study mouse bioassay and qPCR were performed on pig heart digest, while in MC-PCR parasitic DNA was extracted directly from frozen diaphragm tissue, which could influence final concordance results between tests. In the same time, bioassay identifies infectious parasites, while two PCR based techniques target DNA from dead or live parasites. Therefore, the use of two tissues and different objectives for three tests, can explain considerable differences between results. Still the best concordance was observed between bioassay and qPCR confirming the heart as one of the tissues of choice for *T. gondii* analysis in pigs. Compared to bioassay, qPCR failed to detect correctly 16.2% of isolates, showing relatively low sensitivity

(0.74) and specificity (0.84). Nevertheless, qPCR identified correctly majority of truly negative samples (NPV=0.918), but claimed high number of false positive samples (PPV=0.564) (qPCR positive, but bioassay negative). Therefore, if the qPCR on pig heart digest is negative there is 4.6 times higher likelihood that infectious parasites will not be isolated in bioassay. MC-PCR detected accurately *T. gondii* isolates (sensitivity 0.913), but the number of “false negative” samples resulted in poor specificity (0.405). For this study, diaphragms were frozen for several months, while heart digestion, bioassay and qPCR were performed upon the arrival of fresh samples in the laboratory. Although even a short term freezing of meat has a major impact on *T. gondii* infectivity as proven by Hill, Benedetto (103) and Kotula, Dubey (182), there was no impact on preservation of parasitic DNA (183, 184). Moreover, while pork meat, at -25°C keeps its organoleptic characteristics for 12 month because of low activity of present enzymes (185), parasites are concentrated in tissue cysts, which is protected by double membrane. Therefore, the low concordance with both, mouse bioassay (0.36) and qPCR (0.21), as well as low specificity (0.405), positive (0.652) and negative (0.792) predictive values indicate that MC-PCR on pig diaphragm cannot be used in regular diagnostic of *T. gondii*. After experimental infection of 10 pigs with VEG strain (type II), on various muscles 14.1% qPCR and 64.6% mouse bioassays was positive (186), while no PCR and 2 mouse bioassays were positive from retail meat in Mexico (152). Contrary, our study showed 47.1% qPCR and 29.2% of mouse bioassay positives when analysis was performed on the same digested hearts, while 34 pigs were both positive on both techniques (51.5% and 82.9%, respectively). Hears for both bioassay and qPCR were prepared as described in (187, 188), with the goal to liberate bradyzoites from tissue cysts and obtain homogenous number of parasites in the sample (108). Since for the success of bioassay is important that there is infectious dose of live parasites and qPCR results depend upon the presence of parasitic DNA (extracted from live or dead parasites in tissue), one of the explanations is that high numbers of *T. gondii* harboured in pig hearts were dead.

3.5.4. Comparison between indirect and direct detection methods

In this paper MAT, performed on two different matrices (sera and cardiac meat juices), was compared with the direct detection methods (bioassay, qPCR and MC-PCR) as well. The highest Cohen’s kappa values were observed between MAT cardiac juice and direct detection methods (0.66 with bioassay and 0.41 with any direct detection method). Cardiac juice had the best relationship between likelihood ratio, sensitivity and specificity when we compare to mouse bioassay. High negative predictive value of MAT on cardiac fluid ensures identification of truly negative animals. In the same time low positive predictive value explains identification of high number of false positive animals. Similar results had been obtained with ELISA tests or when analysis was tried on sheep samples (54, 91, 189). Positive finding of *T. gondii* specific antibodies by MAT can be regarded as a proof that animal encountered the parasite, but negative bioassay can be consequence of low sensitivity of direct diagnostic methods or parasitic burden which makes infection undetectable. On

the other hand MAT on sera correctly detected truly negative animals (NPV 0.841) but failed to detect around 50% of positive pigs (PPV of sera: 0.46 – dilution 1/6, 0.58 - dilution 1/12, and 0.64 - dilution 1/25). MAT on cardiac juice had better sensitivity and specificity when results of qPCR on heart digest or MC-PCR on diaphragm were used as references. Based on these results the MAT on cardiac juice is considered as “reference” matrix which can be used as a screening test.

3.6. Conclusions

Still, one strain was isolated by mouse bioassay from one pig that was negative in both indirect detection methods (MAT on sera and cardiac fluid) and direct detection method of qPCR. Considering sensitivity and positive predictive values of both matrices (sera PPV=0.456; cardiac fluid PPV=0.696; and diaphragm fluid PPV=0.378) as well as qPCR (PPV=0.564) the error of only one strain is acceptable for MAT and detection of specific DNA. A lack of MAT specificity with cut-off 1/6 can be overcome if the test limit is set to 1/9, as indicated by ROC analysis. However, when using a higher cut-off value, 2 bioassay positive strains would have been assigned to pigs with serological results below the threshold limit (2 isolated strains from pigs with a 1/6), and the meat of these animals would arrive on the public markets as *Toxoplasma*-free.

In summary, the MAT results between different tissues fluids of the same pig differ in concentrations of antibodies, giving the low concordance of the test amongst them. The ideal cut off for all three matrices is 1/9. Sera and cardiac meat juice were highly correlated. However higher specificity and lower sensitivity make sera more suitable for epidemiology research. High negative predictive value makes MAT on cardiac liquid ideal for identification of *T. gondii* – free pigs in public health protection purposes. But, with high level of falsely positives, its suitability is questionable from economic point of view. Still, antibody detection and the presence of parasites are positively correlated, in the sense that with higher titers of antibodies possibility of parasite isolation increases. In the same time, there is a positive correlation between detection of antibodies by MAT and DNA isolation by qPCR. Though moderately correlated with mouse bioassay (kappa value 0.5), qPCR cannot quantify the number of parasites present in the sample. Nonetheless, there is a big difference between meat contaminated with infectious parasites and meat containing specific DNA, and neither current qPCR on heart nor MC-PCR on diaphragm can make distinction. One strain, isolated by mouse bioassay from the animal negative both by indirect and direct detection methods, indicates the potential risk for humans from seronegative pigs as well.

Figure 4 Correlation of MAT on sera and cardiac meat juice in 7 dilutions (1/6, 1/12, 1/25, 1/50, 1/100, 1/200, >1/200).

In grey 45° line through the origin (the concordance line) is represented. Circles represent individual samples (an overlapping is possible represented by a darker colour). Almost perfect correlation is demonstrated between sera and cardiac juice (Pearson's correlation coefficient = 0.85). On each tested dilution there was at least one sample serum positive with negative results of cardiac juice, showing higher sensitivity of sera

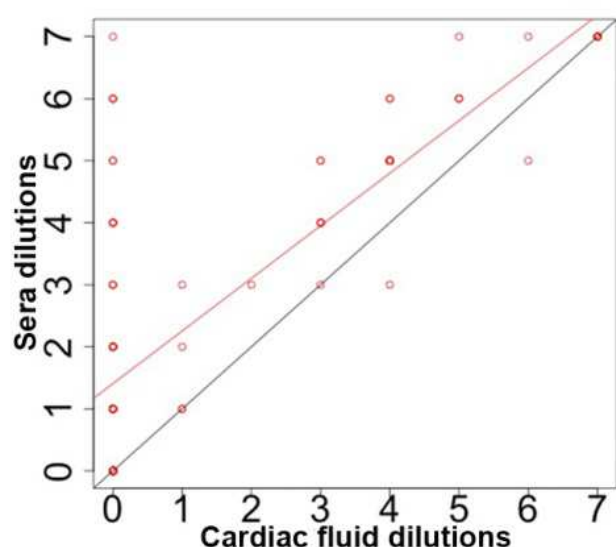


Figure 5 Receiver operating characteristic analysis of MAT result on sera and cardiac meat juice in order to determine the cut off value correlated to parasite isolation in mouse bioassay

The ROC analysis shows that the highest sensitivities and specificities that can be achieved using MAT on any tissue fluid are Se=0.850 (cardiac meat juice) and Sp=0.861 (sera). Nevertheless separate ROC analysis on both pork matrices rappsports the ideal cu off value of 1/9, with highest sensitivity and specificity for cardiac fluid (Se=0.850, Sp=0.829), and high probability value (AUC = 0.888).

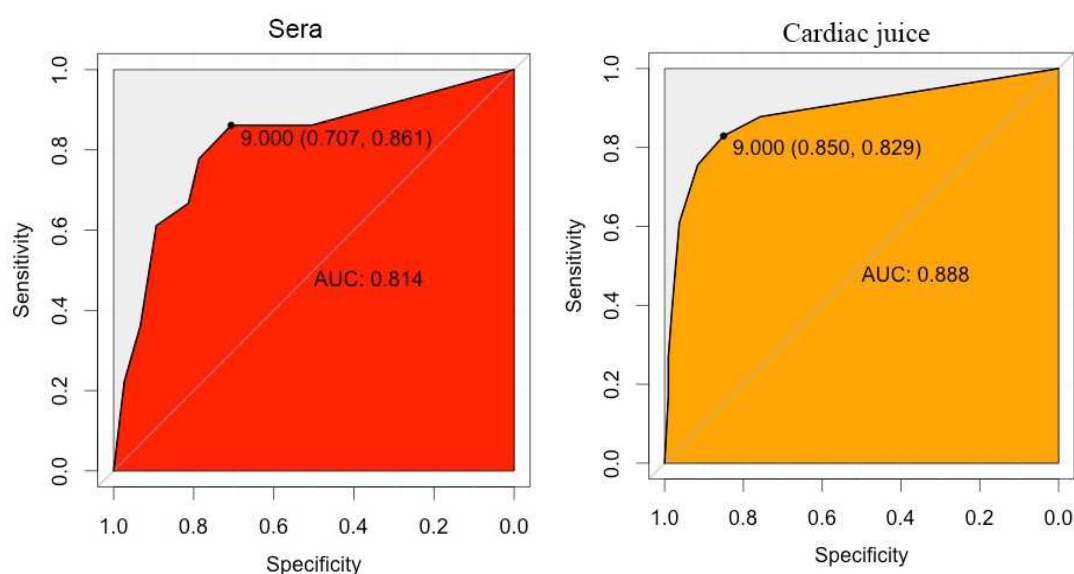


Figure 6 Total number of positive results obtained by direct detection methods in pigs.

Numbers correspond to the numbers of animals found positive at one, two or three methods.

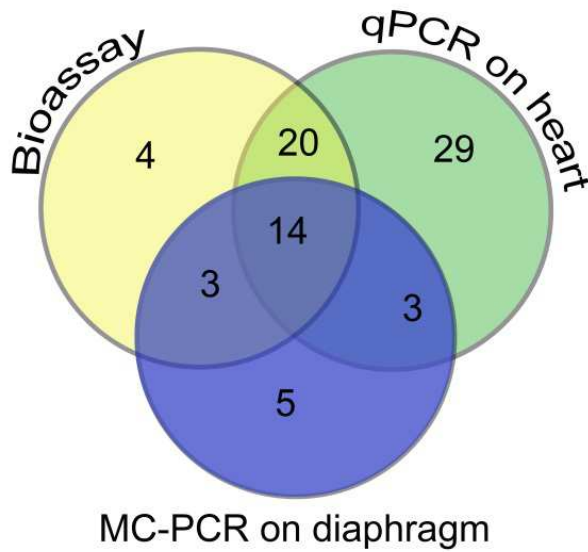


Table 12 Modified agglutination test (MAT) titers and classification (cut-off value $\geq 1:6$) on serum and cardiac juice.

Higher number of positive samples was obtained by sera analysis.

MAT	Serum	Cardiac juice
negative	26	62
positive	72	58
1:6	17	4
1:12	9	1
1:25	7	7
1:50	8	11
1:100	13	4
1:200	8	2
1>200	10	7
Total	98	98

677 **Table 13 Concordance results between detection of anti-*T. gondii* antibodies by MAT from sera, cardiac juice and mouse bioassay, qPCR and MC-PCR.**

678 The table shows highest kappa values, when results of MAT on cardiac juice are compared to parasite isolation in mouse bioassay. Poor concordances have
 679 been observed when diaphragm is used for detection of *T. gondii* antibodies from its fluids or parasitic DNA by MC-PCR.

MAT (IgG)		Mouse bioassay				qPCR				MC-PCR				Any direct detection method			
				Cohen's	95%CI			Cohen's	95%CI			Cohen's	95%CI			Cohen's	95%CI
		neg	pos	kappa		neg	pos	kappa		neg	pos	kappa		neg	pos	kappa	
Cardiac juice																	
1/6	neg	78	5	0.66	0.52-0.80	67	21	0.46	0.31-0.61	79	9	0.23	0.04-0.42	54	29	0.41	0.26-0.58
	pos	14	32	Substantial		15	37	Moderate		36	16	Fair		9	37	Moderate	
1/12	neg	84	6	0.74	0.61-0.87	73	25	0.48	0.33-0.63	89	9	0.33	0.13-0.52	64	34	0.43	0.28-0.58
	pos	8	31	Substantial		9	33	Moderate		26	16	Fair		6	36	Moderate	
Sera																	
1/6	neg	23	1	0.28	0.10-0.45	20	6	0.20	0.01-0.38	22	4	0.05	-0.10-0.20	15	9	0.18	-0.02-0.39
	pos	37	31	Fair		36	36	Poor		55	17	Poor		27	41	Poor	
1/12	neg	38	1	0.52	0.35-0.69	32	11	0.30	0.11-0.48	39	4	0.19	0.02-0.38	29	14	0.36	0.18-0.55
	pos	22	31	Moderate		24	31	Fair		38	17	Slight		17	38	Fair	

706 **Table 14 Sensitivity and specificity of MAT compared to direct detection methods references.**

707 The table shows similar values of sensitivity, specificity, positive and negative predictive values as well as likelihood ratios for all three MAT matrices in first
 708 three dilutions when compared to mouse bioassay, qPCR or MC-PCR. The best results demonstrated MAT on cardiac juice when bioassay was set as a
 709 reference, showing especially high capability to distinguish true negative results (1/6 NPV = 0.940, and 1/12 NPV = 0.933). Moreover, if specific anti - *T.*
 710 *gondii* antibodies are found in a pig cardiac juice on dilutions 1/6 and 1/12, the likelihood for parasite isolation rises 6.27 and 5.63 times, respectively.
 711 Comparison between three direct detection methods shows that qPCR can be used to predict Bioassay results, but not the other way around with high
 712 certainty. MC-PCR demonstrated low sensitivity and specificity values compared to any other *T. gondii* detection method, both direct and indirect.

MAT	Mouse bioassay					qPCR					MC-PCR				
	Se	Sp	PPV	NPV	LL Ratio	Se	Sp	PPV	NPV	LL Ratio	Se	Sp	PPV	NPV	LL Ratio
Sera 1/6	0.383	0.969	0.456	0.958	12.27	0.357	0.857	0.5	0.769	2.5	0.286	0.809	0.764	0.846	1.5
Sera 1/12	0.633	0.969	0.585	0.974	20.27	0.571	0.738	0.564	0.744	2.18	0.506	0.809	0.309	0.907	2.66
Sera 1/25	0.733	0.875	0.636	0.917	5.87	0.696	0.690	0.630	0.75	2.25	0.597	0.714	0.326	0.885	2.09
Cardiac juice 1/6	0.848	0.865	0.696	0.940	6.27	0.817	0.638	0.712	0.761	2.26	0.687	0.64	0.308	0.898	1.91
Cardiac juice 1/12	0.913	0.838	0.795	0.933	5.63	0.890	0.569	0.786	0.745	2.07	0.774	0.64	0.381	1.01	2.15
Cardiac juice 1/25	0.935	0.784	0.829	0.915	4.323	0.915	0.517	0.811	0.728	1.89	0.809	0.6	0.405	0.903	2.02
Bioassay	1	1	1	1	∞	0.919	0.564	0.838	0.739	2.11	0.792	0.652	0.405	0.913	2.28
qPCR	0.739	0.838	0.564	0.918	4.56	1	1	1	1	∞	0.643	0.68	0.293	0.902	2.01
MC-PCR	0.913	0.405	0.651	0.792	1.54	0.902	0.293	0.68	0.643	1.28	1	1	1	1	∞

713 **Table 15 Concordance between direct detection of *Toxoplasma gondii* in pig by mouse bioassay and PCR-based detection of parasitic DNA in trypsin-**
714 **digest of heart, and in 100g of diaphragm by MC-PCR.**

715 Isolation of infectious parasites is more concordant with results of qPCR on heart digest than MC-PCR on diaphragm tissue (Cohen's kappa 0.5 compared to
716 0.36, respectively). In the same time, analysis showed that even if *T. gondii* DNA has not been detected, in 16% cases live parasites can be isolated (Recovery
717 rate 0.16).
718

qPCR	Bioassay		total	Recovery rate	Cohen's kappa	
	negative	positive			Estimation	95%CI
negative	68	6	74	0.16	0.50	0.35-0.66
positive	24	31	55	0.74	Moderate	
total	92	37	129			
MC-PCR						
negative	84	22	106	0.81	0.36	0.16-0.56
positive	8	15	23	0.82	Fair	
total	92	37	129			

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4. Section 4

Working title: *Toxoplasma gondii* antigen for MAT produced in cell culture

4.1. Summary

Toxoplasma gondii is recognized as one of the major foodborne pathogens. Hosts are infected by consumption of tissue cysts present in the meat or ingestion of oocysts from the environment contaminated by cat faeces. Even though animal or meat inspection for *T. gondii* does not exist anywhere in the world, it is important to have reliable method for parasite's diagnostic in epidemiology purposes, and risk assessment for public health protection. The aim of this study was to replace the mouse experimentation with cell cultures and standardize numbers of tachyzoites for Modified agglutination test antigen (MAT). Production of *T. gondii* Rh tachyzoites was evaluated in i) Human Foreskin Fibroblasts (HFF); ii) human colon ileocecal colorectal adenocarcinoma (Hct-8); iii) human epithelial colorectal adenocarcinoma cells (Caco-2). Their sensitivities and specificities were compared with antigen produced in mice, as described by Desmonts and Remington in 1980, on pig, cat, dog, sheep, goat sera, as well as on bovine cardiac fluids. CaCo-2 cell culture flasks were destroyed within hours after infection, and antigen from Rh tachyzoites, amplified in HCT-8 cell lines, gave agglutination reaction difficult to observe. A total of $2-4 \times 10^8$ fixed tachyzoites per flask of HFF cell lines was obtained (1-2 ml of antigen). The lowest Cohen's kappa concordance between two antigens was observed when pork sera were tested (Cohen's kappa: substantial - 0.64), while almost perfect values were shown on goat, cat sera, and bovine cardiac fluid (Cohen's kappa: 0.81; 0.81, 0.82, respectively). Experimentally infected mouse and sheep sera, demonstrated perfect concordances between two antigens. Pearson's product moment test showed substantial correlation (0.78, 95%CI: 0.71-0.84) when antigens were compared on pig sera, while all other samples showed perfect correlations throughout all 8 dilutions. In this study we replaced mice with HFF cell lines, for *T. gondii* Rh amplification for MAT antigen, and demonstrated reliable method that can be used in routine diagnostic of parasite infection. Furthermore, production of HFF cells and passage of Rh tachyzoites does not require highly trained staff or special facilities, and can be performed in laboratories equipped with standard cell culture devices.

4.2. Introduction:

The parasite from phylum *Apicomplexa*, *Toxoplasma gondii*, infects all warm blooded animals rendering the most successful parasite on Earth (190, 191). *Toxoplasma gondii* is recognized as one of the major foodborne pathogens. The parasite's life cycle includes i) sexual phase developed only in the intestines of felids (definitive hosts) which results in shedding of environmentally resistant oocysts and ii) asexual stages, when parasite circulates in nature without genetic recombination (clonal division) between prey and predators (intermediate hosts). Hosts are infected by consumption of tissue cysts present in the meat or ingestion of oocysts from the environment contaminated by cat faeces (192). Replication and activity of the parasite is controlled by the immune response of the host (193). Although generally mild and self-limiting in immunocompetent individuals, *T. gondii* infection may cause life-threatening disease in the foetus and in the immunocompromised individuals.

Even though animal or meat inspection for *T. gondii* does not exist anywhere in the world, it is important to have reliable method for parasite's diagnostic in epidemiology purposes, and risk assessment for public health protection. For the diagnostic of *T. gondii* in animals, as a source of human infection, serological test is mostly used. Sabin Feldman dye test has been a gold standard for *T. gondii* specific antibody detection for years, but since the live parasites are used, only reference laboratories can perform this analysis (41). Desmonts and Remington (1980) described Modified Agglutination test (MAT), widely used for detection of antibodies specific for this parasite ever since. MAT has been used for investigation of serologic status in cattle (41), pigs (43-45), sheep (46, 47), chickens (48), dogs and cats (50), as well as humans (52, 53). Recently MAT has been used for detection of antibodies in meat destined for human consumption as well (54).

Antigen for MAT consists out of whole *T. gondii* tachyzoites, fixed in formalin. Desmonts and Remington inoculated mice intra-peritoneally with mouse TG 180 sarcoma cells infected with *T. gondii* Rh strain (type I). This method enabled collection of ten times more parasites in comparison with simple passage through animals (42). Two major requirements need to be fulfilled in order to obtain antigen with satisfactory quality: i) intra-peritoneal exudate needs to be harvested when almost all sarcoma cells are heavily infected, close to disruption and a great number of free parasites that appear morphologically normal (alive) are observed; ii) mice should be inoculated between 48 - 72h before their exudate is collected. These two requirements are difficult to achieve resulting in careful production of antigen in only few specialized laboratories.

The first description of *T. gondii* cultivation in cell cultures dates from 1953 (194). Different cell cultures were mainly used for research of parasite biology, morphology and different life stages, immune cell responses and in research of potential vectors and reservoirs (Table 16).

Successful production and use of live parasites from HeLa cells for dye test was described by (195), but the continuous use of parasites was proved as not stable due to unpredicted growth and loss

of antigenic markers (196) (Table 6). Hassl and Aspöck described purification of *T. gondii* bradyzoites by percol gradient and fixation in formaldehyde in order to produce antigen for indirect immunofluorescent antibody test (197). Finally in 1991 Suresh et al succeeded to isolate proteins from Rh strain in Vero cell culture and use them for ELISA and IFAT tests (198). Moreover, their experiment showed no alterations of antigenic determinants of parasites even after long-term passages of parasites in cell cultures and no need of specific parasite purification from cells. Parasite production for diagnostic purposes in dye test was described as late as 1973, but little was done on improvements (199). First rapport of agglutinating antigen produced in Vero cells was by (200), but the parasite yields were too low and technique was never improved (Table 16).

The aim of this study was to replace the animal experimentation with cell culture and better standardize production of MAT antigen. For this purpose, the use of three cell lines as a source of *T. gondii* Rh tachyzoites was evaluated. The antigen for MAT was produced using the Rh strain tachyzoites amplified in three cell cultures: i) Human Foreskin Fibroblasts (HFF); ii) human colon ileocecal colorectal adenocarcinoma (Hct-8); iii) human epithelial colorectal adenocarcinoma cells (Caco-2). Their sensitivities and specificities were compared with antigen produced in mice as described by Desmonts and Remington. We analysed antigens performances on sera samples from pigs, cats, dogs, sheep, goats, as well as on bovine cardiac fluids obtained from surveillance projects of *Toxoplasma gondii* in our laboratory. Cheap, efficient and applicable method for antigen production available to numerous laboratories would facilitate and improve identification of contaminated meat, enhancing prevention of human infection.

4.3. Materials and Methods:

4.3.1. Antigen definition:

Antigen for MAT is comprised from the whole *T. gondii* tachyzoites. Parasites were fixed crude in formalin and stored in antigen buffer.

4.3.2. Mouse antigen preparation:

Mouse antigen was prepared according to Desmonts and Remington (1980) (Table 16). In short 2x10⁶ *T. gondii* tachyzoites of Rh strain were inoculated i.p. into each female Swiss Webster CD I mouse (4 weeks old) with 5x10⁴ TG 180 sarcoma cells. After two days, mice were sacrificed and intraperitoneal exudate was harvested, mixed with new 2x10⁴ sarcoma cells (obtained from mice infected only with these cells, without parasites). Forty-eight to 72 hours later intraperitoneal fluid was collected, centrifuged and digested in trypsin (Table 16). Digested cells and liberated parasites were

washed in PBS and centrifuged after which fixation in formalin was done for 16-20 hours. Fixed parasites were washed three times in PBS, counted and diluted in alkaline buffer in concentration of 2×10^8 / ml (Table 16). Sodium-Azid buffer was added to store the antigen.

4.3.3. Cell culture antigen formulation:

Three different cell cultures were tested separately in this experiment. Flasks of 75ml with: i) human foreskin fibroblast (HFF) cells, ii) human colon ileocecal colorectal adenocarcinoma (Hct-8) and iii) human epithelial colorectal adenocarcinoma cells (Caco-2) were prepared independently according to Wu et al (2009). When reached 95% of confluence, cell flasks were inoculated with 2×10^6 Toxo Rh strain tachyzoites. Type I, Rh strain is fast replicating strain in almost any type of tissue culture, cloning at the rate of $\approx 119/48h$. After 48 - 96 hours parasites were harvested from flasks and antigen was prepared according to the procedure described in Table 16. The protocol, established by Desmonts and Remington, was improved by replacing the mice with cell cultures for amplification and preparation of *T. gondii* Rh tachyzoites. In brief, $2 - 5.5 \times 10^7$ of parasites/ml was collected from flasks two days after inoculation, when 95-100% of cells were destroyed. Tachyzoites were washed twice in PBS in order to eliminate cell debris, cell medium leftovers and broken parasites. Fixation was then done for minimum 16 hours in formaldehyde. Two washings before and after fixation in PBS were determined as optimum limiting parasite loss. Fixed tachyzoites were counted and diluted in alkaline antigen buffer in adjusted concentration of 2×10^8 per ml (Table 16).

By using the cell culture for amplification of *T. gondii* Rh tachyzoites there was no need to use two generations of parasites and sarcoma cells (Table 2. Steps 2, 3 and 4) in contrast to description of Desmonts and Remington when antigen was produced in mice.

4.3.4. Samples for testing

Comparison between two antigens was performed on bovine cardiac fluids, sheep, pig, goat, dog and cat sera. In 2009, bovine cardiac fluids were collected in French slaughterhouses for National surveillance study of *Toxoplasma gondii* in cattle (11). Three lambs, three month old, were experimentally infected p.o. with 10^3 oocysts of ME49 strain (type II), in order to follow parasite kinetics in sheep. For this study, sera samples taken on the day 52 P.I. were used, when all animals still had high titer of antibodies. Pig sera and cardiac fluids came from pigs raised and slaughtered in France and were collected for National surveillance plan conducted in 2013 (108). Dog sera originated from French military dogs stationed in Senegal as well as dogs found in different villages in Senegal near French military bases (201). Cat sera were collected from domestic cats admitted for different medical conditions to veterinary school small animal hospital in Maisons-Alfort (CHUVA, ENVA)

from 2012 until today. Goat sera were collected for virology tests from one goat farm on island of Corsica in 2014 (courtesy of Dr. Emmanuel Bréard).

4.3.5. Modified agglutination test

Modified agglutination test was performed according to Desmonts and Remington (42). Initial dilution - 1/3, of sera and cardiac fluids were made in PBS. In testing plate eight two-fold dilutions were made, starting from 1/6 to 1/800, and 25µl from the last well was discarded. All antigens were diluted separately in Bovine Albumin Buffer Solution (BABS) in 1/17 ratio. On separate plates 25µl of antigen was distributed in each well. Sixteen to 20 hours later, the plates were observed for agglutination. For all tested antigens, plates were prepared in the same time, as well as sample dilutions. In order to avoid biased results, the same examiner repeated each MAT analysis two times. Blind tests, performed by different/various technicians from laboratory, were also organized.

4.3.6. Statistical analysis

All statistics were performed using the R Core Team (2013) (113). First, χ^2 test was used to determine independence of the results. The McNamara's test calculated the differences in sensitivity between the antigens. To calculate the concordance between tests on qualitative level (negative/positive), taking into account accordance by chance, Cohen's kappa test was used (fmsb package). Six different concordance categories were determined as described by Landis and Koch (158): i) < 0.00: no concordance; ii) 0.00 – 0.20: poor; iii) 0.21- 0.40: fair; iv) 0.41 – 0.60: moderate; v) 0.61 – 0.80: substantial; vi) 0.81-1.00: almost perfect. In order to calculate correlation between two antigens on all dilutions (quantitative values), Pearson's product moment correlation coefficient was used (R stats package). The same agreement interpretation categories were used as for Cohen's kappa test.

4.4. Results:

4.4.1. Antigen production

CaCo-2 cell culture flasks were destroyed within hours after infection even when smaller parasite dose (6×10^3) was used for inoculation. Obtained parasites did not preserve the shape and their proliferation results were negligible, which made them unsuitable for MAT antigen production. When Rh tachyzoites, amplified in HCT-8 cell lines, were used for MAT antigen, agglutination reaction was difficult to observe, even though there was no change in parasite's morphology.

Agglutination could be read easily when HFF cell culture was used for parasite amplification. A total of $2-4 \times 10^8$ fixed tachyzoites per 75ml flask was obtained, which was sufficient for 1-2 ml of antigen (concentration of 2×10^8 /ml). Further results present the comparisons between antigens produced from mouse intraperitoneal exudate and HFF cells.

In order to evaluate newly prepared antigen, sera were used from ii) naturally infected pigs, goats, cats, and dogs; ii) bovine cardiac fluids ii) sera from experimentally infected sheep and mice.

4.4.2. Concordance:

The Cohen's kappa showed excellent concordance between antigens produced from mice and the one produced from HFF cultured parasites (Cohen's kappa between 0.64 - 1) (Table 17). The lowest concordance between two antigens was observed when pork sera were tested (Cohen's kappa substantial - 0.64, 95%CI: 0.4-0.9). Almost perfect concordance was shown on goat, cat sera, and bovine cardiac fluid since mouse antigen yielded higher numbers of seropositive animals (Cohen's kappa: 0.81 95%CI: 0.65-0.99; 0.81 95%CI: 0.64-0.97; 0.82 95%CI: 0.65-0.99, respectively) (Table 17). Dog sera and all controls, demonstrated perfect concordances between antigens produced in mice or in cell culture (Cohen's kappa 1.00, 1.00, 1.00, respectively). McNamara test validated statistically substantial similarities between antigens for all samples (McNamara p-value between 0.074-NA), except for pig sera (p-value: 0.013). Highest differences are observed on pork samples but still not statistically significant (Table 17).

4.4.3. Correlation:

Mouse antigen showed higher sensitivity on 1/6, 1/50, and cell culture formulation was more sensitive on 1/25 dilutions, however without statistical significance. When higher dilutions were compared, no statistically significant difference was observed. In six cases, cell culture antigen showed lower value, but allowed identification of seropositive animals (Table 18). Pearson's product moment test showed substantial or almost perfect correlations for all tested matrices (Correlation between 0.78-0.96). The substantial correlation (0.78, 95%CI: 0.71-0.84) was observed when antigens were compared on pig sera, while all other samples showed perfect correlations throughout all 8 dilutions (Table 18).

4.4.4. Cost-benefit:

Taking into account animal reduction and replacement, prices for animal facilities and time necessary for parasite passages the price goes to favour of cell culture estimated to $\approx 20-25$ €/ml in contrast to ≈ 30 €/ml when mice are used (Table 16).

4.5. Discussion

Although the serological positive animal does not mean it harbors infectious parasites; farm/region/country prevalence is a good indicator of meat contamination with *T. gondii* (88, 202).

Cell cultures were used to study parasite biology, infectivity for different tissues and reduce animal experiments for parasite propagation and conservation (Table 19). Later research tried to replace bioassays, but the susceptibility of cell cultures to bacterial and fungal infection showed that in vitro models are not appropriate for routine diagnostic purposes (203-205). When CaCo-2 were infected even with 10^3 Rh tachyzoites, cell culture was destroyed in 24 hours with no significant proliferation of parasites (number of parasites doubled). HCT-8 would be the best choice for large scale tachyzoites replication. Because of the balanced replication of parasites and cell growth, the same flask could be used twice for parasite production. When those parasites were used in MAT antigen, neither positive nor negative reaction could be observed easily. In positive reaction agglutination web was very thin, almost non-recognizable, which could not be improved even with higher concentrations of parasites in antigen. Also, agglutination “button” could not be seen in negative controls. This was probably due to a changed density of fixed tachyzoites, or parasites maybe lost some of their antigenic characteristics after continuous passages as described by Mavin, Joss (196). We confirmed that HFF were the easiest cell line for proliferation of *T. gondii* Rh as earlier was described (206, 207). We then, successfully purified and fixed tachyzoites for MAT antigen production. Moreover, from HFF cell culture flasks, numbers of tachyzoites were more constant (around $2-4 \times 10^7$ /ml after 48h). Out of three tested cell lines, the best production of Toxo antigen was when Rh tachyzoites were amplified in HFF cells. Both correlation and concordance results between antigens produced in HFF and in mice showed high levels of agreements. Finally, MAT test showed characteristic species - independency when antigen formulated with *T. gondii* parasites grown in HFF cells was used.

In order to evaluate newly constructed antigen, sera and cardiac fluids from naturally and experimentally infected animals were tested for *T. gondii* specific antibodies by MAT. Results were compared with MAT outcomes obtained with conventional mouse antigen. Our results indicate that antigen from mice showed higher prevalence, especially for bovine tissue fluid. This difference can be explained by the immune characteristics of tissue fluids described by Forbes, Parker (62), as antibodies are not concentrated equally in all parts of the matrix. Nevertheless, these differences in prevalence between two antigens are not statistically significant. Mainly in lower dilutions (1/6, 1/12) mouse produced antigen found more positive results, while in higher (1/50, 1/100, 1/200, 1/400) both identified same seropositive animals. High concordances between two antigens were calculated when MAT on animal sera was compared. Almost perfect correlations were shown when two MAT antigens



were compared on naturally infected bovine cardiac fluids, sera from pigs, goats, cats and dogs as well as on experimentally infected sheep and mice. Cohen's kappa between two antigens showed almost perfect agreement, confirming that use of HFF cell cultures is possible for constant production of parasites for agglutination test.

The use of Modified agglutination test for detection of *T. gondii* specific antibodies showed always better results (sensitivity, specificity) than other types of serology tests (ELISA, IFAT, LAT, ect). Only recent studies, with newly constructed ELISA antigens, conducted on swine and chicken sera and pork tissue fluids, showed lower sensitivity and specificity of MAT (48, 145). Moreover, Hamidinejat, Nabavi (48) compared MAT and ELISA results with *T. gondii* specific DNA findings in PCR from chicken tissues in receiver operating characteristics analysis (ROC) and found higher sensitivity and specificity for ELISA test. But authors of both studies did provide neither the details of how the MAT antigen was prepared nor the results of control samples. On the other hand, Forbes, Parker (62) compared commercial ELISA, in house MAT, PCR and mouse bioassay on sera and tissue fluids of experimentally infected pigs and saw higher concordances between MAT and direct detection techniques. The animals with high parasitic burden and strong immune answer are easy to detect, but, so far, MAT always succeeded to identify the animals with low titers of antibodies and parasites in tissues, having higher concordances with both PCR and bioassay.

In this study we proved that it is possible to replace mice with HFF cell lines, for production of MAT antigen. Antigen from parasites amplified in HFF cells is stable, reliable and can be used in routine diagnostic of *T. gondii* infection. Furthermore, production of HFF cells and passage of Rh tachyzoites does not require highly trained staff or special facilities, and can be performed in laboratories equipped with standard cell culture devices. Production of MAT antigen in different laboratories can be a source of unequal sensitivities and specificities. To validate reproduction of results, quality controls should be organised for batches of antigen, internally as well as by national references centres and laboratories.

Table 16 Differences between MAT-antigen produced in mice and cell culture.

For production of antigen in mice, for one harvest two sets of mice are needed (steps 1-5), whereas only one inoculation and parasite collection after two days is sufficient for parasite replication in cell culture. Tachyzoites are collected when 95-100% of cells are destroyed in cell culture. On the other hand, parasites from mouse peritoneal liquid should be collected when 50% of the carcinoma cells liberated *T. gondii*, and 50% are still infected with large numbers of tachyzoites. By amplifying the Rh tachyzoites in cell culture there is no need of culture/tissue digestion, thus the loss of parasites is smaller.

Procedure		A) Mouse antigen production 	B) Cell culture antigen production 
1	Inoculation with $2 \cdot 10^6$ parasites	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
2	Inoculation with $5 \cdot 10^4$ TG 180 sarcoma cells	<input checked="" type="checkbox"/>	<input type="checkbox"/>
3	Euthanasia and collection of infected sarcoma cells with parasites	<input checked="" type="checkbox"/>	<input type="checkbox"/>
4	Inoculation of new mice with parasite infected sarcoma cell and new $2 \cdot 10^4$ TG 180 sarcoma cells	<input checked="" type="checkbox"/>	<input type="checkbox"/>
5	Collection of parasites	<input checked="" type="checkbox"/> *	<input checked="" type="checkbox"/>
6	Centrifugation at 600g for 10 minutes	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
7	Digestion with 5% trypsin 2 minutes at 37°C	<input checked="" type="checkbox"/>	<input type="checkbox"/>
8	Washing in PBS and centrifugation at 600g for 10 minutes	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
9	Fixation of parasites in 6% Formaldehyde for minimum 16 hours	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
10	Centrifugation at 600g for 10 minutes	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
11	Washing in PBS and centrifugation at 600g for 10 minutes 3X	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
12	Fixed and washed parasites are transferred into alkaline buffer (NaCl, H ₃ BO ₃ , NaOH, Bovine plasma albumin, fraction V, distilled water)	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
13	Number of the parasites in antigen is adjusted and dilution of the antigen determined	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
14	Adding of the 0,1% Sodium Azide	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>

*Parasites should be collected 2-3 days after infection in 3rd or 4th stadium when half of the parasites destroyed their cells, and half is still inside sarcoma cells.

Table 17 Quantitative comparison between MAT antigens produced in mice and derived from cell cultured *Toxoplasma gondii* tachyzoites

Two antigens were compared on pig, goat, cat and dog sera, bovine cardiac fluids and mice and ovine sera as controls. McNemar test of similarity shows that seroplogy results from all species are similar except for pig sera. However Cohen's kappa test calculated substantial concordance between results obtained with two antigens for pig sera and almost perfect agreement for all other types of samples. All control and dog sera showed perfect concordances.

Samples	Antigen from cell culture		Antigen from mice		Total nb of samples	Chi-square	McNemar test of similarity		Cohen's kappa test of concordance beyond chance			
	Neg	Pos	Neg	Pos		<i>p</i> -value	X^2	<i>p</i> -value	κ		95% Confidence interval	
Control Mice	4	4	4	4	8	<0.001	NA	NA	1,00	Perfect	1,000	1,000
Control Sheep	1	6	1	6	7	<0.001	NA	NA	1,00	Perfect	1,000	1,000
Pig Sera	148	8	140	16	156	<0.001	6,125	0,013	0,64	Substantial	0,401	0,884
Bovine CF	36	14	32	18	50	<0.001	2,250	0,134	0,82	Almost perfect	0,646	0,989
Goat Sera	27	25	32	20	52	<0.001	3,200	0,074	0,81	Almost perfect	0,644	0,968
Cat Sera	41	9	40	10	50	<0.001	0,000	1,000	0,81	Almost perfect	0,591	1,019
Dog Sera	24	26	24	26	50	<0.001	NA	NA	1,00	Perfect	1,000	1,000

Table 18 Qualitative comparisons of MAT results when mouse and cell culture antigens are used

Eight different dilutions, from 1/6 to 1/800, were used for the correlation comparison. Two antigens showed higher agreements on very low (1/6, 1/12) and very high (1/200, 1/>200) dilutions. Pearson's product moment correlation showed almost perfect correlation for all analyzed matrices ranging from 0.78 in pigs (95%CI: 0.712-0.836) and 0.94 in dogs (95%CI: 0.898-0.966). Bovine cardiac fluid, although with unstandardized concentration antibodies also showed almost perfect correlation (0.80, 95%CI: 0.674-0.883). Mouse sera and sheep sera from experimentally infected animals all showed perfect correlations between two antigens (0.96 and 0.94, respectively).

Samples	Number of samples with correlated results in MAT, analyzed with Antigens from cell culture and from mice on 8 two-fold dilutions									Total number of samples	Pearson's product-moment correlation		
	Neg	6	12	25	50	100	200	>200	Σ of correlated		<i>r</i>	95% Confidence interval	
Control Mice	5	0	0	0	0	1	3	2	11	12	0,96	0,849	0,988
Control Sheep	1	0	2	0	0	0	0	4	7	7	0,94	0,898	0,966
Pig Sera	140	0	0	0	0	2	0	0	142	156	0,78	0,712	0,836
Bovine CF	32	8	2	0	0	0	0	0	42	50	0,80	0,674	0,883
Goat Sera	27	0	0	0	0	0	14	0	41	52	0,93	0,885	0,961
Cat Sera	39	0	0	0	0	0	6	0	45	50	0,89	0,808	0,934
Dog Sera	24	4	0	0	0	0	22	0	50	50	0,94	0,898	0,966

Table 19 Review of the use of different cell cultures in *Toxoplasma gondii* research

The table shows the use of different cell lines and cultures for *T. gondii* propagation, parasite infection diagnostic purposes and studies of life cycle. The first use of cell culture was published in 1953 for research of *T. gondii* parasitemia. In later years cell cultures were mainly used for studies of parasite biology, interaction with different tissues and immune systems responses to infection. Later studies from 1990s try to use cell cultures in direct detection purposes and compare them with PCR and mouse bioassays. In recent years cell cultures became a source of parasites for serology analyses and specific protein isolation.

Year	Cell culture	Use	publication
1953	blood	Parasite biology	(194)
1963	No data available	No data available	(208)
1965	Rat retina	To study the interaction between <i>T. gondii</i> Rh strain and cells of neuroectodermal origin	(209)
1966	Avian embryos	Propagation of Rh strain	(210)
1967	Chicken fibroblasts	To study the differences between <i>T. gondii</i> strains	(211)
1968	PC-Y-15 from swine kidney BS-C-1 from monkey kidney SIRC rabbit corneal epithelium	Use of different cell culture lines for propagation and freezing of <i>T. gondii</i>	(212)
1968	Human amniotic cells	Propagation of Rh strain in amniotic cells	(213)
1969	No data available	No data available	(214)
1974	No data available	No data available	(215)
1970	No data available	No data available	(216)
1970	Monkey kidney cell	Sporozoite biology	(217)
1971	Guinea-pig kidney cell Mouse L-cell tissue	Propagation and virulence changes in avirulent strains	(218)
1973	Mosquito cell lines, vero cells	Potential role of mosquitos as vectors for <i>T. gondii</i>	(219)
1973		Isolation of <i>T. gondii</i> in cell culture	(199)
1974	Human macrophages from peripheral blood	Macrophage activity to <i>T. gondii</i> infection	(220)
1975	Human diploid fibroblasts, WI-38	Impact on human immune system	(221)
1978	Human larynx carcinoma cells	Mass replication of parasites in cell culture	(222)
1979	Baby hamster	Purification of Rh tachyzoites by	(223)

	kidney cells	filtration through glass-wool filtering fibre	
1980	Lamb testicular cells	Propagation and preservation of Rh strain in cell culture in order to replace mice.	(224)
1982	Plant protoplasts, HeLa cells	Attempt to infect plant cells with parasite	(225)
1985	L-cells	Diagnostics of Toxoplasmosis- case rapport.	(226)
1991	bovine monocyte, human fetal lung, and Madin-Darby bovine kidney cell cultures	Tissue cyst formation	(227)
1992	Fibroblast cell culture	Comparison of sensitivity and efficacy of the use of cell culture and bioassays in mice and rabbits	(203)
1993	MRC5	Tachyzoite-Bradyzoite interconversion	(228)
1994	HFF	Tachyzoite-bradyzoite interconversion – triggering mechanisms, and stage specific protein identification	(207)
1995	MRC5 cell line	Comparison of PCR, Giemsa staining and cell culture assay in Toxoplasmosis diagnostics from AIDS patients	(205)
1995	HFF	Bradyzoite development of ME49	(229)
1996	MRC-5 cell line	The sensitivity of detection of a wild-type strain of <i>Toxoplasma gondii</i> by cell culture, mouse inoculation, and PCR was determined	(204)
2000	HeLa cell culture	Rh tachyzoite production for Dye test	(195)
2004	HD11, MQ-NCSU - chicken macrophage cell lines	Chicken macrophage interaction with Rh strain tachyzoites	(230)
2004	HeLa cell cultures	Comparison of three lineages of <i>Toxoplasma gondii</i> RH strain in terms of performance in the dye test, culture, and gene expression	(231)
2009	HFF, HeLa	Study of <i>Toxoplasma gondii</i> RH strain tachyzoites proliferation in human foreskin fibroblast (HFF) cells and HeLa cells.	(206)
2010	HeLa	Comparisons of <i>Toxoplasma</i> strains propagation in the HeLa cell culture and animals	(232)
2011	HeLa	Study aimed to determine a novel cell culture strategy for <i>T. gondii</i> RH Ankara	(233)

	strain tachyzoites to harvest abundant tachyzoites with least host cell contamination and minimal antigenic variation at predetermined dates to use as an antigen source in serological assays that will facilitate reduction in animal use.	
2011 Vero-E6	To investigate parasite-host dynamics in cultures of <i>Toxoplasma gondii</i>	(234)
2012 Vero cells	Study was designed to determine whether <i>T. gondii</i> tachyzoites grown in Vero cell cultures in serum-free medium, after many passages, are able to maintain the same antigenic properties as those maintained in experimental mice.	(235)
2012 induced pluripotent stem cell (iPSC)-derived culture systems	Development of cell cultures for the research of cell and developmental biology, immunology, pharmacology	(236)

General discussion and conclusions

Pork production and sampling problems

In 2013 World food consumption of meat was 42.4 kg/capita, which makes an increase of 10% in the past ten years (FAO., 2015). This growth was followed with increase in meat production from 2.4×10^8 to 3.1×10^8 tons, while human population increased in the same period from 6.4 billion to 7.2 billion people. In European consumption had almost no increase from 73.1 to 76.01kg/ capita, which followed the growth in population numbers for the past 10 years, as well. In developing countries consumption of pork was higher than production (consumption growth rate = 2.1%, production growth rate = 2.0%). EU countries used these opportunities for their increase in pig production which followed exponential growth in piglet numbers, although the numbers of sows and gilts changed in lesser extent (EU Commission - Directorate-General for Agriculture and Rural Development – Short Term Outlook – N°13). In 2007 EU Commission issued new sets of rules to which all pig producers had to compel by the January 2013 (Council Directives 2007/834/EC and 2008/889/EC). Pig producers and slaughterhouses got under a great pressure by these conditions, while in the same time, they were obliged to satisfy new regulations and compete in foreign markets with pork producers from developing countries where production laws and rules still have not met same standards (EU Commission - Directorate-General for Agriculture and Rural Development – Short Term Outlook – N°13). In the past years even “small” losses, meant farm closing and loss of a producer, while constant growth in pork demand was recorder on world markets (Directorate-General for Agriculture and Rural Development – Short Term Outlook – N°13). In this environment the role of veterinary services was to protect the pig production as well as public health. The aim of this project was to describe seroprevalence of the world’s most disseminated parasite – *Toxoplasma gondii* in pigs produced in France, identify on farm risk factors for animal infection, evaluate existing detection methods and asses the risk for public health protection.

In 2010, 5,700 pig farms was registered only in Bretagne region in France (237). In those farms 7.4 million pigs was raised in 2014. In the same period, in Bretagne 13 slaughterhouses were active (counting with 73,300 employees) (237). This means that average slaughtering speed, during 252 working days, was 283 pigs/hour. In some abattoirs this number could even reach 2000 animals/hour. In order to accomplish necessary efficiency and obtain as much as possible produce from each animal, almost all slaughterhouses use “hollow knives” system for bleeding the animals. In this system hollow needles are placed in pig’s jugular veins and vacuum pumps suck out the blood from unconscious animal carcass. This method allows blood collection and conservation from all animals together as aseptically as possible. However, the speed of the slaughter line, as well as cumulative collection of blood, makes sera sampling in epidemiology purposes very difficult or even

impossible. Therefore, to avoid this problem, tests that can be used on other tissue/s are necessary for *T. gondii* screening of pigs.

Breeding type is important for *T. gondii* prevalence in pigs

In the review of Toxoplasmosis in pigs over the last 20 years, Dubey (10) published the table (Table 1) of seroprevalence across the World. Pigs of all age categories and from all breeding systems were analysed. In different studies, various diagnostic tests with even greater variety of cut off values were published, and therefore comparisons between results could not be made. From this table, three patterns are distinguishable: i) seroprevalence of pigs in free-range and organic farms are considerably higher than those from indoor systems; ii) seroprevalence rises with age; iii) in the period from 1989 to 2009 seroprevalence of *T. gondii* in pigs were lowered globally. Only eight, out of 78 studies analysed in this table, reported risk factors analysis for *T. gondii* infection in pig farms. In USA access of young cats to pigs, lack of rodent control, outdoor housing and farms size were determined as major risk factors in four different studies (238-241). The data about production type (indoor/outdoor/organic) or farm risk factors are missing, because majority of the studies sampled pigs directly at the slaughter line in the abattoirs. Therefore Dubey (10) identified only two studies in which intensity of *T. gondii* infection on intensive pig farms was analysed. These two surveys found prevalence of 0.38% in Netherlands and 32.1% in Panama (87, 242). These differences are probably consequence of general trend that pigs from Central and South America are more infected irrespective the breeding method.

Amongst outdoor bred pigs in Europe, in the last 15 years, seroprevalence ranged from 5% in Netherlands to as high as 25% in outdoor sows from Romania and Switzerland (Table 4. in introduction). One of the explanations for such a high differences can be the level of contact with wild life, especially rodent populations. Diversity of species and numbers of rodents are higher in mountainous forests of Switzerland and west Romania than in the plains of Netherlands. Since no surveys so far have been done on wild life from these countries this explanation is only presumption. In European intensively bred pigs the prevalence are considerably lower, between 0.38% in Netherlands to 16.6% in Spain (Table 4). Such a high levels of *T. gondii* contamination in pigs from Spain Garcia-Bocanegra, Simon-Grife (86), explain by high presence of cats and absence of rodent control on 20-25% of the farms, respectively. On the other hand, study in Netherlands does not even discuss the possibility that rodent control lacks on intensive pig farms.

Nation-wide study on slaughtered pigs in France showed prevalence of 3% in intensively breed and 6.2% in outdoor animals. Moreover, from 1342 analysed intensive pigs, 25 different strains were isolated from finishers and sows. This means that 1.9% pigs (1.2% of fattening pigs, and 5.2% sows), originating from intensive farms in France, slaughtered for human consumption, are *T. gondii*

carriers. In France, production of pork from outdoor farms is destined for small, regional markets and traditional products, making output of around 15% in 2013 (243). In our study from 207 outdoor pig samples, 16 (7.7%) harboured *T. gondii* parasites (7.7% of fattening pigs, and 14.3% sows). Usually older animal tissues (e.g. sows) are used for delicatessen such as sausages, different types of salami, hams, bacon, etc., while meat from young animals (piglets and fattening pigs) is sold fresh. General opinion is that the risk of acquiring *T. gondii* infection is higher when pork is consumed after insufficient thermal treatment (145). Inadequate salting, drying, pressure treatment or packaging of meat products can be a source of infectious parasites as well, especially when we consider that live *T. gondii* was isolated from 5.2% of intensively and 14.3% outdoor bred sows.

The ranges of animal infection with *T. gondii* throughout Europe were reported in 87 papers (Tables 2, 3 and 4). Out of those, parasite prevalence was described in 16 papers from 14 countries for pigs grown in intensive farms, in 8 publications from 6 EU members for outdoor animals and seroprevalence in wild boars was described in 10 countries including the closed ecosystem on island of Corsica (France) (Figure 3). In general, prevalence of *T. gondii* in pigs is well studied in west Europe (Netherlands, France, Portugal), while from centre or east of the continent we lack data either for intensive or outdoor pig farms. Only in Switzerland was reported higher seroprevalence from intensive than outdoor pig farms. All other countries described prevalence considerably lower in indoor kept animals. Intensity of infection above 50% was found only in outdoor animals from Austria. Contrarily, in Netherlands prevalence from all breeding types were less than 5%, as well as in wild boar. Nonetheless, pigs from intensive farms were the least infected, while wild animals had the highest intensity of *T. gondii* contamination. In the Netherlands, France, Portugal, Sweden, Spain and Latvia seroprevalence in wild boar is higher than in domestic pigs from both breeding systems. Interestingly in Switzerland and Romania, seroprevalence in wild boar is lower than in outdoor pigs. This can be explained by rough climate, which kills oocysts in soil, and population of rodents is found more frequently around food stocks on outdoor or even intensive farms.

Quantitative prediction of pork as an important source for human infection

In Europe out of 76.01kg of meat /per capita consumed in 2013, 34.7kg was pig meat. In 2013 France produced 5.5×10^6 tons of meat, out which 2.1×10^6 tons was pork. Pork represents the major meat consumed in France with 33.5kg/per capita/year. With *T. gondii* prevalence of 3% in pigs from intensive farms, and 6.2% in outdoor-bred animals, it is important to analyse the risk of acquiring infection by pork consumption.

As described previously, results from nation-wide studies of three major production species (pigs, sheep, cattle) support the fact that seropositive animal is not automatically carrier of infectious *T. gondii*. In pig study from intensive farms out of 963 fattening pigs, in 22 cases specific anti - *T. gondii*

antibodies were found. From those animals 11 strains were isolated, which calculates 0.5, sensitivity of mouse bioassay when compared to MAT results. Similar ratio was found in intensively bred sows as well, where 25 seropositives 13 harboured parasites infectious to mice (sensitivity = 0.52). In outdoor - pigs this ratio is higher, when 15 strains were isolated from 16 seropositive fattening pigs (sensitivity 0.94), and 1 strain was isolated from 2 seropositive sows (7 outdoor sows in total were sampled which is not enough for further analysis).

French abattoirs in 2103 slaughtered 24,396,000 pigs out of which 4,915,000 were piglets (up to 2 month of age or 25kg weight (Agreste., 2015). On a domestic market in France nearly 2.1×10^6 tones of pork are sold, divided between 1.2×10^5 tones of piglet meat and 1.9×10^6 tones of fattening and reproduction animals pork. Out of these numbers 98.5% of pigs were grown in intensive farms while only 1.5% originated from outdoor farms. In nation-wide study no *T. gondii* strain was isolated from piglets, therefore further analysis will concentrate on fattening pigs and sows. The same study found adjusted 2.9% prevalence of *T. gondii* in 5,337,193 intensively bred fattening pigs, and 13.4% in 13,851,592 sows. This means that 154,779 fattening and 1,856,113 reproduction animals have detectable concentrations of anti – *T. gondii* antibodies. When sensitivity of bioassay (0.5) is taken into account, we can calculate a total of 1005446 intensively bred pigs in France that harbour *T. gondii* parasites. Average weight of fattening pigs at the abattoir is 115kg (110-140kg), from which animal carcass after slaughter weights around 86kg. From obtained meat 25-30kg are cut for different produces and 55-60kg are sold as fresh pork. This means that on French markets 86,468 tons of pork infected with *T. gondii* was sold. Hence out of total intensively bred pork, consumed in France, 4.1% harboured infectious *T. gondii* parasites. In the same time, 1.5% of French pork market consists of meat from outdoor-bred animals. Nation-wide study found 6.3% adjusted prevalence for 101,028 fattening pigs from outdoor farms. Calculated sensitivity of bioassay in outdoor pigs is 0.94, which computes 5983 animals that harbour infectious *T. gondii*. From 5983 animals 514.5 tones of meat was sold to a consumers. Therefore, out of total number of outdoor raised pigs destined for human consumption, 5.9% were infected with live *T. gondii* tissue cysts. This proves that consumption of pork originating from outdoor farms exposes humans in higher extent to *T. gondii* infection. However, pork from intensive farms is more represented in French cuisines, and infected pieces count for 4.1% of all pig meat consumed in country. Whereas infected meat from outdoor-bred animals, accounts only for 0.2% of total meat consumed in France. In 2013, France had 65525000 habitants (244), and pork consumption was 33.5 kg/capita (Faostat03, 2015). Therefore, there is 20.4 times higher probability that humans in France were in contact with pork contaminated with *T. gondii*, originating from indoor than from outdoor farms.

Nevertheless, this calculation does not mean that there is 20 times greater chance of acquiring *T. gondii* infection if pork from intensive farms is consumed in contrast to outdoor raised animals. For that type of prediction a complex set of data still lack. In order to calculate probability of obtaining the

143 *T. gondii* infection from intensive or outdoor-bred animals, we need to calculate emission and
144 exposition rates as well as effect of contamination.

145 To calculate emission rates it is necessary to estimate sensibility of the analysis – number of
146 tissue cysts/parasites found in comparison with real number of *T. gondii* present in the analysed tissue.
147 In Toxoplasma research, parasite and parasitic DNA isolation from animals have been expressed as
148 qualitative values (positive/negative). Even though name suggests quantification process, not even
149 qPCR has been able to quantify numbers of isolated parasites. Therefore, it is impossible to estimate
150 concentration of parasites in tissues of experimentally and naturally infected animals. Consequently,
151 we lack information about tissue distribution of *T. gondii* in pigs. Jurankova, Basso (91), proved that
152 brain is the predilection site, followed by lungs and heart. Edible tissues were ranked fourth.
153 Nonetheless, the distribution of *T. gondii* throughout specific tissues has not been studied. Therefore
154 we cannot tell if the cysts are concentrated in one part of the tissue (more likely) or they have
155 homogenous distribution. This information is of crucial importance in order to show if the ingestion of
156 each piece of meat bears the same risk of infection.

157 The exposition rate represents the relation between the intake of contaminated meat and
158 concentration of parasites present in the edible tissues. For majority of animal species each country
159 can calculate quantity of meat consumed per capita per year (e.g. for pork in France it is
160 33.5kg/capita/2013). In order to express the levels of meat contamination it is necessary to calculate
161 the number of infectious parasites. Since mouse and cat bioassays are qualitative tests, from which it is
162 impossible to quantify infectious parasites in examined tissues. PCR based detection techniques can
163 quantify parasitic DNA presents in the tissues, but they are still not able to differentiate between dead
164 and live parasites.

165 In order to quantify the risk for infection with any pathogen, it is necessary to establish dose-
166 response relation. Data from experimental studies about different doses of various strains used for pig
167 infection are numerous as well as for other animal species (60, 62, 112, 125, 156, 245). However, in
168 none of these studies the lowest infectious dose of any strain has been shown. In the same time, to
169 calculate risk of human infection, we need data about human dose response to various strains, but
170 those data lack as well. We can argue that for immuno-compromised categories one tissue-cyst of any
171 strain is sufficient for infection and development of disease. Still, majority of population is consisted
172 of immunocompetent individuals, for which different *T. gondii* strains have variable infectivity.

173 One of that has not been taken in consideration for this analysis is type of meat used in human
174 nutrition. In general it has been considered that sow meat is used more often for cured products, while
175 meat from younger categories has been sold fresh for further treatment (145). Various reports across
176 the world suggest that around 10% of pork delicatessen has not been cured sufficiently (106, 140, 246,

247) Therefore inappropriate use of curing methods and poor thermic processing of fresh meat cuts represent risk for human infection.

Because we are interested in risk of acquiring *T. gondii* infection by consuming pig meat produced in France, complex analysis of import/export trends was not taken into account.

Prevalence of *T. gondii* in intensive pig farms from Bretagne: comparison between two studies

Our risk factors study from Bretagne showed that pigs get infected with *T. gondii* more frequently on farms with more than 2000 animals, as well as in small households with other species. The same results were observed in USA (145). In the same time, it showed that clothes and boot changing before entering different pens does little to protect animals against the parasite, but Danish entry system installed on buildings prevent direct contact of cats or rodents with pigs and consequently *T. gondii* infection. Therefore, the only sources of *T. gondii* infection of pigs from heavily contained intensive farms from Bretagne can be the food or water supplies and dispensers. *T. gondii* oocysts are resistant enough to survive various purification processes and can be found in wells, ponds, lakes and even in tap water (248). Dubey, Weigel (249) reported that major source of infection for pigs on farms in Illinois were opened wells. Besides water sources, cats can shed oocysts into farm food stocks where, if not eliminated within 2-5 days, they sporulate and become infectious for pigs (180, 250). Rodent carcasses are often found in open silo or food stocks, and they can be a source of various diseases for pigs (251), along with *T. gondii* in the form of tissue cysts. Davies, Morrow (252) showed that if pigs are bred in strict containment in North Carolina, *T. gondii* infection is reduced to sporadic cases.

It may seem that overall *T. gondii* prevalence of 6.9% in intensively bred pigs in Bretagne region of France is higher than one calculated in Nation-wide study in 2013. For the risk factor analysis and prevalence in intensively bred pigs in Bretagne, farms were randomly chosen based on the annual numbers of animals (finally 500 suckling piglets were sampled, 389 weaned piglets, and 2532 fattening pigs). Whereas for French national study, regions were chosen based on the annual numbers of slaughtered pigs and each was represented with the same number of samples (Bretagne with 35 piglets, 150 fattening pigs, 70 sows samples from intensive farms). From pigs on intensively bred farms examiners collected sera directly. For nation-wide study heart and blood samples were collected by abattoir veterinarians. In order to avoid possibility that heart and sera samples were mismatched in national study, cardiac fluids were used for prevalence analysis. In the same time, 37% of sera were missing from Bretagne, therefore seroprevalence could be calculated for 35 piglets, 61 fattening pigs and 64 sows (total=160 animals). Straight a way it is evident that Bretagne is under

represented in national study, especially when we considered that 92% of all pork produced in France originates from this region. Collection of more samples from Bretagne would over-represent this region and mask prevalence on country level, which was avoided by stratified sampling in this study. With same numbers of samples from each region, prevalence was analysed among slaughtered pigs on the country level. Two age categorisations were used: i) Bretagne study - organisation of farm and fattening cycle: suckling, weaned piglets and fattening pigs; ii) National study - age categories slaughtered in abattoirs: piglets, fattening pigs, sows. Risk factor analyses across the World rapport age as one of the main risk factor for infection, as in longer lifespan animal has more chances to encounter the parasite (78, 86, 118, 253-255). We found no reason why would France be an exception, which was proven in nation-wide study. In national study no strain was isolated from MAT positive piglets, neither on sera nor on cardiac fluids, which indicates presence of maternal antibodies (91). In the same time, risk factor analysis showed no statistically significant difference between levels of *T. gondii* infection in piglets and fattening pigs. Therefore, the goal was to find in which step of breeding pigs are the most vulnerable to *T. gondii* infection and when infection occurs.

When prevalence for fattening animals was calculated (only category in common), 6.4% *Toxoplasma* seropositive pigs were found in Bretagne study in contrast to 1.6% seropositive and 3.3% of cardiac fluid positives in national study. If two piglet categories from Bretagne survey would be merged together, obtained seroprevalence is 8.5% in contrast to 0%; and 2.9% prevalence of cardiac fluid in national study (Table 20). One of the explanations can be low number of samples from Bretagne collected in nation-wide survey. When prevalence is low, in order to confirm it, the number of collected samples should be higher (Thrusfield., 2007), otherwise standard error becomes non acceptable. When the number of samples is low as intensive pig sera in nation-wide study, confidence intervals are considerably higher. When compared 95% confidence interval extremes, results in two studies are more similar. The sera for study of *T. gondii* in pigs from Bretagne were collected from 2006 – 2008, while collection of samples for national study was done in 2013. One of the possibilities is that as well as in other countries, by improving the pig breeding methodology, zoo-technical and – hygienic conditions on farm, infection rate dropped in just several years (145), which would make obtained results comparable.

Table 20 Comparison between *Toxoplasma gondii* prevalence in pigs from Bretagne obtained in Bretagne study and in Nation-wide parasite surveillance of pork

	Bretagne study (2006-2008)				Nation-wide study – only samples from Bretagne (2013)		
	Suckling Piglets	Weaned Piglets	Piglets (Suckling + Weaned)	Fattening Pigs	Piglets	Fattening pigs	Sows
Cardiac fluids	NA	NA	NA	NA	35	150	70
Prevalence (%)	NA	NA	NA	NA	2.9	3.3	14.3
Standard error					0.028	0.015	0.042
95% Confidence interval	NA	NA	NA	NA	-2.7 – 8.5	0.4 – 6.2	6.1 – 22.5
Sera samples	500	389	889	2532	35	61	64
Seroprevalence (%)	7	10.5	8.5	6.4	0	1.6	12.5
Standard error	0.011	0.016	0.009	0.005			
95% Confidence interval	4.8 – 9.1	7.4 – 13.6	6.7 – 10.3	5.4 – 7.4	0.000	-1.5 – 4.7	4.5 – 20.5

Comparison between *T. gondii* prevalence in pigs, sheep and cattle, three national surveillance plans in France

Results from literature about seroprevalence analyses in production animal (Tables 3 and 4) show extensive *T. gondii* infection of all domestic species. Sheep are the most infected, with prevalence ranging from 15% in lambs from France and 27.8% in adults from the Netherlands, to 84.5% in Serbia and 89% in France. Sheep are bred mainly on pastures, which makes them the most exposed to environmental contamination with *T. gondii* oocysts. Even if kept inside (during winter months), sheep are fed with dried hay and little or none of other food is used. In contrast to sheep nutrition, dairy cows and lactating goats besides pasture and hay are fed with concentrated nutrients. This can explain slightly lower prevalence of *T. gondii* in these two species in contrast to sheep. Lowest prevalence in goats was recorded in Portugal (18.5%) while highest infection rates were found in Serbia (73.3%). In cattle, lowest seroprevalence was found in Portugal as in goats (7.5%) while highest were reported in Spain (83.3%)(Table 3). While seroprevalence in all three ruminant species vary greatly throughout Europe, it is still discussed if the meat, meat products or milk from these animals represents danger for public health. Meat from sheep and goats as well as their delicatessen are well treated in salts or well boiled in order to make it tenderer, while the lamb or goat kid meat in some countries is prepared half cooked (“agneau rose” in France for example). Until today, only one study isolated live *T. gondii* from clinically healthy cattle in nation-wide study in France, when heart samples were collected on the slaughter line (11). In the same time, numerous studies report about isolation of the parasites from milk and milk products of all ruminants, especially goats (256-261). From literature data about *T. gondii* occurrence in ruminants we can only hypothesise that infection extent is directly related to breeding methods and levels of its development from country to country. In Spain for example, cattle are ranches in semi extensive conditions and most of the time animals spend on pastures, same as goats. In the same time in ex Yugoslavian countries goats were forbidden species for over 30 years, and animals have been imported in the last 10 years in Serbia, and seroprevalence is high as a result of low developed breeding methods. In Europe, equine infection with *T. gondii* is reported in 5 countries. The lowest seroprevalence was recorded in horses from Sweden, while highest intensity of infection was found in horses from Romania (Table 3). When considered that only in France, Switzerland, Italy and Japan horsemeat is eaten raw or semi cooked the risk of acquiring infection can be reduced to these countries. Isolation of infectious *T. gondii* was successful from experimentally infected animals, but data about naturally infected animals are scarce and not well studied.

In France, first nation-wide study was done on sheep meat by Halos, Thebault (47). MAT was done on cardiac fluids. The overall seroprevalence calculated in lambs was 17.7%, and 89% in adults.

Region wise, there was an increase from northwest to southeast in lamb samples but no such conclusion could be made for adults. In total 46 strains were isolated, from which one was genotyped by RFLP as type III and the rest as type II (47). In total, 16 districts of sheep production in France were identified. Unlike to nation-wide study in pigs, where administrative region production was used to determine representative regions (producing 96% of country's pork), 16 districts that produced 100% of sheep meat were modelled in seven areas (Table 21) (47). For this comparison, first we grouped two breeding types (intensive and outdoor) and then the pig production regions were reorganized in order to match geographical areas of sheep sampling. In piglets as well in fattening pigs and sows, neither region of origin nor of slaughtering was statistically important for *T. gondii* prevalence. The same can be observed when administrative regions are regrouped to match sheep sampling strategy. The observed increase of *T. gondii* prevalence in lambs could not be seen for piglets or for other two age categories (Table 21). The highest number of strains isolated from lambs (6 strains) was from Pyrenees mountains area, as well as from piglets (11 strains) (Aquitaine and Midi-Pyrenees regions in pig study). No strain was isolated from lambs slaughtered in Western and mid-eastern areas, while in piglets 6 and 7 strains were found, respective to regions (West: Bretagne, Basse Normandie, Pays de la Loire; mid-east: Auvergne). In adult sheep and pigs no pattern can be observed (Table 21). While *T. gondii* prevalence in sheep depends upon the pasture and hay contamination with oocysts, therefore upon the numbers of domestic and wild felids; in pigs (especially from intensive farms) this can be a consequence of multiple factors. Indoor pigs, if kept in strictly confined conditions, have less chance to be contaminated with *T. gondii* oocysts than sheep, and if the on farm rodent control is conducted properly, risk to encounter tissue cysts in infected mouse or rat is lower. Although pigs from outdoor farms are bred in similar conditions as sheep (which is confirmed with higher *T. gondii* prevalence in this breeding type), these animals are still fed by concentrated nutrients and factory prepared food. This can explain lower prevalence in outdoor pigs compared to sheep, especially if we consider that for experimental infection of sheep minimum of 200 oocysts is needed, while in pigs, ingestion of 10 oocysts developed clinical signs of disease (180, 262).

310 **Table 21 Comparison of seroprevalence and parasite isolation from sheep (2009) and pigs (2013) in France.**

311 This table compares results of two national studies conducted in France. Sheep had been analysed in 2008 (47), and pigs in 2013 (Djokic, et al., 2016). For
 312 sheep study production areas had been determined based on the number of produced animals in 2007. In pig study administrative regionalisation had been
 313 used, and the sample size was determined based on the annual number of animals slaughtered (from each age category) per region.

Production areas/Regions		Sample size (young/reproductively capable)		Mean Prevalence in animals <1year		Mean Prevalence in animals >1year		Strain isolation				
Sheep	Pigs*	Sheep	Pigs*	Sheep	Pigs*	Sheep	Pigs*	Total	Sheep <1year	Pigs* <1year	Sheep >1year	Pigs* >1year
Western area	Bretagne,	50 (40 : 9)	703 (563 : 140)	0.10	0.02	0.82	0.14	22	0	6	4	12
	Basse Normandie											
	Pays de la Loire											
Mid- Western area	Poitou-Charentes	101 (90 : 11)	43 (38 : 5)	0.11	0.03	1.00	0	12	5	1	6	0
Pyrenees mountains area	Aquitaine Midi-Pyrenees	49 (36 : 13)	515 (427 : 88)	0.32	0.04	0.68	0.07	25	6	11	6	2
Mid-Eastern area	Auvergne	50 (45 : 5)	245 (220 : 25)	0.14	0.05	1.00	0.04	9	0	7	2	0
Central Area	Centre	50 (37 : 13)	43 (43 : 0)	0.12	0.07	0.84	NA	8	1	2	5	0
Northern Area	NA	25 (19 : 6)	NA	0.21	NA	0.83	NA	2	0	NA	2	NA
South-East Area	NA	101 (75 : 26)	NA	0.24	NA	0.59	NA	11	2	NA	9	NA

314 NA = Not Analysed ; Pigs* = results from two age categories, piglets and fattening pigs, observed as one.

315

Nation-wide study of *T. gondii* infection in beef produced in France was conducted in 2009 (11). In total 2351 samples were collected in the slaughterhouses throughout the whole country, out of which 572 were hearts of young categories (younger than one year) and 1779 samples from animals older than one year. MAT was performed on cardiac fluids. Overall *T. gondii* prevalence in young age categories was 4.9% while in older cattle was 24.5%. More interestingly this was the first study to isolate infectious *T. gondii* from clinically healthy bovine destined for human consumption. In young cattle categories, prevalence is lower than in piglets (Table 22), except in animals from Midi-Pyrenees and Centre regions (although from Centre only 9 calves were sampled). The highest prevalence in adult beef was recorded in Centre region (73.2%) and lowest in Auvergne (16.4%). When compared to pigs, the extents of *T. gondii* infection in cattle are more similar to outdoor than to intensive pigs. Still, this prevalence is higher than infection rates calculated for pigs from any analysed region in France. In order to obtain the best possible quality beef, in France, cattle are bred mainly on pastures or in semi enclosed conditions and concentrated nutrients are provided mainly during winter months. As well as sheep, cattle change pastures regularly in order to obtain enough of the green nutrients. This makes animals more exposed to *T. gondii* infection through ingestion of oocysts. In contrast, outdoor pigs are reared in conditions that support natural behaviour, but are mainly fed with concentrated nutrients in order to obtain desired weight as soon as possible. By pasturing and foraging the ground pigs destroy the meadow, and therefore are kept in the same enclosed space around the main farm facilities throughout the seasons. On the other hand, intensively bred animals are kept in confined pens for their whole life and contact with possible *T. gondii* contamination sources is close to nothing. The data from French national study indicate that older bovines are more prone to infection. Still, the question why *T. gondii* strains are scarcely found mainly in animals with clinical signs of toxoplasmosis or in animals with reproduction problems remains unanswered.

Table 22 Comparison of *Toxoplasma gondii* seroprevalence from cattle and pig studies in France

The table compares results of two national studies conducted in France. Cattle study was done in 2009 (11, 47), and pigs in 2013 (Djokic, et al., 2016). In total 2351 cattle samples was analysed, out which 572 animals younger than one year and 1779 of old age category. Overall prevalence in calves was 4.9%, and 24.5 in adult bovines. Cattle from all administrative regions of France were for sheep study production areas had been determined based on the number of produced animals in 2007. In pig study administrative regionalisation had been used, and the sample size was determined based on the annual number of animals slaughtered (from each age category) per region.

Areas of 92% production of pork in France	Pigs – positives/total Prevalence (%)						Cattle – positives/total Prevalence (%)	
	Intensive farms			Outdoor farms			Young (<1 year)	Adults (>1year)
	Piglets	Fattening pigs	Sows	Piglets	Fattening pigs	Sows		
Aquitaine	0	7/222 (3.2)	0	0	1/36 (2.8)	0	NA	NA
Auvergne	3/40 (7.5)	3/148 (2.0)	1/25 (4.0)	0	5/32 (15.6)	0	0/33 0	11/67 16.4
Basse-Normandie	0	3/182 (1.6)	0	NA	NA	NA	0/31 0	26/119 21.8
Bretagne	1/35 (2.9)	5/150 (3.3)	10/70 (14.3)	0/5 (NA)	0/3 (NA)	0	0/131 0	57/269 21.2
Centre	NA	NA	NA	0	3/43 (7.0)	0	5/9 55.6	30/41 73.2
Midi-Pyrénées	0/3 (NA)	3/137 (2.2)	4/86 (4.7)	0	5/29 (17.2)	2/2 (1.0)	4/77 5.2	18/73 24.7
Pays de la Loire	0/50 (0.0)	1/124 (0.8)	10/70 (14.3)	0	1/14 (7.1)	0	0/23 0	17/96 17.7
Poitou-Charentes	NA	NA	NA	0	1/38 (2.6)	0/5 (0.0)	NA	9/50 18.0
Total	4/128	22/963	25/251	0/5	16/195	2/7	9/304	168/714

Comparison between *T. gondii* prevalence in pigs and other two meat producing species (sheep and cattle), show lower rates of infection in both intensive and outdoor animals. Pig breeding practices and nutrition sources differ greatly to those used in sheep and cattle breeding, and offer more protection against parasite infection. Therefore, the only finding of similarity in infection extents between lamb and piglet infection levels in Midi-Pyrenees region can be regarded as inadvertent. High differences in prevalence, but almost the same numbers of isolated *T. gondii* strains between sheep and pigs (46 and 41) goes to show that the second one are more susceptible to infection with this parasite. This can be due to a fact that pigs do not have fore stomachs so fewer parasites are destroyed in digestion. In the same time, parasitaemia in pigs lasts for 2-4 months after infection, while in sheep there is no trace of parasites in circulation after only a few days (120, 263, 264). This means that either pigs are evolved to be more prone to *T. gondii* infection, or their immune system is “slower” in answering the parasite invasion.

Can MAT be useful tool for screening of pigs for *T. gondii* infection

In literature, different ELISA tests showed the highest sensitivities and specificities for *T. gondii* diagnostic in pigs. Bokken, Bergwerff (61) devised Bead – Based Assay (BBA), which was highly concordant with results of 3 different ELISA, but so far test performance has not been compared to direct isolation of *T. gondii* or parasitic DNA. In the same time, serologically positive animal does not always harbour the parasites (44, 60, 265-268). However, the seroprevalence of *T. gondii* can be a good indicator of meat contamination (Berger-Schoch, et al., 2011, Hamilton, et al., 2015). The levels of concordance between detection of *T. gondii* specific antibodies and isolation of infectious parasites or parasitic DNA have been tested in few studies of MAT and in house ELISAs (44, 48, 62, 156, 269). Majority of newly developed tests have high concordances between each other or with MAT and DT test, but what is the level of agreement with PCR or bioassay results has not been analysed (60, 112, 131, 161, 268).

For detection of anti – *T. gondii* specific antibodies in tissue fluids, MAT was the test with highest sensitivity and specificity, (49, 62). One of the essential differences that give MAT advantage, is the use of whole, formalin fixed tachyzoites as an antigen. Desmonts and Remington (42) described the method for MAT antigen production from intra-peritoneally infected mice with the help of mouse TG sarcoma cells. Although very effective, this method is time consuming, expensive and requires highly experienced personnel. This limited the use of MAT only to a handful of laboratories capable for producing the antigen in sufficient quantities. In order to facilitate the accessibility of MAT we formulated the antigen out of *T. gondii* tachyzoites grown on HFF cell culture.

In our antigen production we replaced the amplification of parasites in mice with cell culture in order to improve standardization of the production. As described by Wu, Zhang (206), HFF were

the easiest cell culture for proliferation of *T. gondii* Rh and parasite purification. Rh-ERP strain was chosen for two major reasons: i) the same strain was used by Desmonts and Remington; ii) it is a fast replicating strain that kept its virulence to mice even after 77 years of constant passages in laboratory conditions (270). Although Mavin, Joss (231) showed that when HeLa cell culture was used for *T. gondii* Rh -B, -J and - Q strains cultivation, parasitic gene expression was not always stable, making them not suitable for DT test and after 30 propagations in HFF cells, no changes were observed in Rh-ERP strain. Moreover, when mouse inoculation with 10 Rh-ERP tachyzoites propagated in HFF cell culture was tried they died after 10 days, exactly as mice infected with parasites maintained in experimental animals. Our study showed high correlation between mouse and cell culture antigen in 8 tested dilutions when all five animal species were compared.

This study showed high kappa concordance between antigens produced from HFF cultured parasites and from mouse peritoneum grown sarcoma cells. This proved MAT as the test of choice when screening cattle, goats, cats, dogs, and pigs for the presence of *T. gondii* - specific antibodies. When different dilution results were compared, the differences between mouse and cell culture antigen were even smaller. Moreover with standardised protocol for MAT antigen production in HFF cells, test can be easily available in any laboratory with basic equipment for cell line growth.

Our analysis proved that MAT is a valuable tool for identification of *T. gondii* infected pigs. We showed that the ideal cut off dilution for testing of pig sera and cardiac fluids in MAT is 1/9, with high probabilities (AUC: 0.814 and 0.888, respectively) for isolation of infectious parasites from hearts of serologically positive animals. With sensitivity of 0.913 and specificity of 0.838 on cardiac fluids MAT identified 100% of animals *T. gondii* negative in mouse bioassay. Even with sensitivity of 0.633 and specificity of 0.969 on pig sera, MAT negative predictive value is 0.974, which makes it ideal tool in identifying truly *T. gondii* negative pigs. In contrast low positive predictive values of MAT suggest that test identifies high number of false positive pigs, which may cause high economic losses. Bearing in mind low sensitivity of MAT on pig sera and difficulties to obtain blood samples in abattoirs, we suggest the use of cardiac fluids as a matrix of choice for animal screening purposes. MAT analysis of cardiac fluids is capable to identify Toxoplasma-free pigs with 93% accuracy, without the need to use additional direct detection methods and can be applied as standardized screening method on the slaughter lines throughout the world.

List of references:

1. Tenter AMH, A. R.; Weiss, L. M. *Toxoplasma gondii*: from animals to humans. *Int J Parasitol.* 2000;30(12-13):1217-58.
2. Cook AJ, Gilbert RE, Buffolano W, Zufferey J, Petersen E, Jenum PA, et al. Sources of toxoplasma infection in pregnant women: European multicentre case-control study. *European Research Network on Congenital Toxoplasmosis. BMJ.* 2000;321(7254):142-7.
3. Bobic B, Nikolic A, Klun I, Vujanic M, Djurkovic-Djakovic O. Undercooked meat consumption remains the major risk factor for *Toxoplasma* infection in Serbia. *Parassitologia.* 2007;49(4):227-30.
4. Markovic M, Iovic V, Stajner T, Djokic V, Klun I, Bobic B, et al. Evidence for genetic diversity of *Toxoplasma gondii* in selected intermediate hosts in Serbia. *Comparative immunology, microbiology and infectious diseases.* 2014;37(3):173-9.
5. Esteban-Redondo I, Maley SW, Thomson K, Nicoll S, Wright S, Buxton D, et al. Detection of *T. gondii* in tissues of sheep and cattle following oral infection. *Vet Parasitol.* 1999;86(3):155-71.
6. Hemsworth PH, Barnett JL. Behavioural responses affecting gilt and sow reproduction. *Journal of reproduction and fertility Supplement.* 1990;40:343-54.
7. Decision No 1482/2007/EC of the European Parliament and of the Council of 11 December 2007 establishing a Community programme to improve the operation of taxation systems in the internal market (Fiscalis 2013) and repealing Decision No 2235/2002/EC, (2007).
8. Früh B. Organic Pig Production in Europe - Health Management in Common Organic Pig Farming. Jacqueline Forster GW, editor 2011.
9. Commission Decision of 18 November 2008 amending Decisions 2002/747/EC, 2003/31/EC, 2005/342/EC, 2005/344/EC and 2005/360/EC in order to prolong the validity of the ecological criteria for the award of the Community eco-label to certain products (notified under document number C(2008) 6941) (Text with EEA relevance) (2008).
10. Dubey JP. Toxoplasmosis in pigs--the last 20 years. *Vet Parasitol.* 2009;164(2-4):89-103.
11. Blaga R, Aubert D, Perret C, Geers R, Djokic V, Villena I, et al. Animaux réservoirs de *Toxoplasma gondii* : état des lieux en France. *Revue Francophone des Laboratoires.* 2015;2015(477):35-52.
12. Fritz HM, Bowyer PW, Bogyo M, Conrad PA, Boothroyd JC. Proteomic analysis of fractionated *Toxoplasma* oocysts reveals clues to their environmental resistance. *PLoS One.* 2012;7(1):e29955.
13. Dumetre A, Dubey JP, Ferguson DJ, Bongrand P, Azas N, Puech PH. Mechanics of the *Toxoplasma gondii* oocyst wall. *Proceedings of the National Academy of Sciences of the United States of America.* 2013;110(28):11535-40.
14. Dubey JP. *Toxoplasma gondii* oocyst survival under defined temperatures. *The Journal of parasitology.* 1998;84(4):862-5.
15. Dubey JP. The history of *Toxoplasma gondii*--the first 100 years. *The Journal of eukaryotic microbiology.* 2008;55(6):467-75.
16. Dubey J, Beattie C. Toxoplasmosis in man (homo sapiens). In: Dubey J, Beattie C, editors. *Toxoplasmosis of animals and man.* Florida: CRC Press, Inc.; 1988. p. 41-60.
17. Lyons RE, McLeod R, Roberts CW. *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. *Trends in parasitology.* 2002;18(5):198-201.
18. Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. *Int J Parasitol.* 1998;28(7):1019-24.
19. Dubey JP, Lindsay DS, Speer CA. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. *Clin Microbiol Rev.* 1998;11(2):267-99.
20. Song C, Chiasson MA, Nursimulu N, Hung SS, Wasmuth J, Grigg ME, et al. Metabolic reconstruction identifies strain-specific regulation of virulence in *Toxoplasma gondii*. *Molecular systems biology.* 2013;9:708.
21. Dubey JP, Frenkel JK. Feline toxoplasmosis from acutely infected mice and the development of *Toxoplasma* cysts. *The Journal of protozoology.* 1976;23(4):537-46.
22. Frenkel JK, Dubey JP, Miller NL. *Toxoplasma gondii* in cats: fecal stages identified as coccidian oocysts. *Science.* 1970;167(3919):893-6.

23. Bushkin GG, Motari E, Carpentieri A, Dubey JP, Costello CE, Robbins PW, et al. Evidence for a structural role for acid-fast lipids in oocyst walls of *Cryptosporidium*, *Toxoplasma*, and *Eimeria*. *mBio*. 2013;4(5):e00387-13.
24. Lindsay DS, Collins MV, Mitchell SM, Cole RA, Flick GJ, Wetch CN, et al. Sporulation and survival of *Toxoplasma gondii* oocysts in seawater. *The Journal of eukaryotic microbiology*. 2003;50 Suppl:687-8.
25. Lelu M, Villena I, Darde ML, Aubert D, Geers R, Dupuis E, et al. Quantitative estimation of the viability of *Toxoplasma gondii* oocysts in soil. *Appl Environ Microbiol*. 2012;78(15):5127-32.
26. Howe DK, Sibley LD. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *The Journal of infectious diseases*. 1995;172(6):1561-6.
27. Levine ND. Progress in taxonomy of the Apicomplexan protozoa. *The Journal of protozoology*. 1988;35(4):518-20.
28. Su C, Khan A, Zhou P, Majumdar D, Ajzenberg D, Darde ML, et al. Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(15):5844-9.
29. Sibley LD, Ajioka JW. Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. *Annu Rev Microbiol*. 2008;62:329-51.
30. Khan A, Dubey JP, Su C, Ajioka JW, Rosenthal BM, Sibley LD. Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *Int J Parasitol*. 2011;41(6):645-55.
31. Ajzenberg D, Banuls AL, Su C, Dumetre A, Demar M, Carme B, et al. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *Int J Parasitol*. 2004;34(10):1185-96.
32. Mercier A, Devillard S, Ngoubangoye B, Bonnabau H, Banuls AL, Durand P, et al. Additional haplogroups of *Toxoplasma gondii* out of Africa: population structure and mouse-virulence of strains from Gabon. *PLoS neglected tropical diseases*. 2010;4(11):e876.
33. Su C, Evans D, Cole RH, Kissinger JC, Ajioka JW, Sibley LD. Recent expansion of *Toxoplasma* through enhanced oral transmission. *Science*. 2003;299(5605):414-6.
34. Zeder MA. Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(33):11597-604.
35. Ajzenberg D, Banuls AL, Tibayrenc M, Darde ML. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol*. 2002;32(1):27-38.
36. Su C, Zhang X, Dubey JP. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. *Int J Parasitol*. 2006;36(7):841-8.
37. Bacci C, Vismarra A, Mangia C, Bonardi S, Bruini I, Genchi M, et al. Detection of *Toxoplasma gondii* in free-range, organic pigs in Italy using serological and molecular methods. *International journal of food microbiology*. 2015;202:54-6.
38. Dias RA, Navarro IT, Ruffolo BB, Bugni FM, Castro MV, Freire RL. *Toxoplasma gondii* in fresh pork sausage and seroprevalence in butchers from factories in Londrina, Parana State, Brazil. *Revista do Instituto de Medicina Tropical de Sao Paulo*. 2005;47(4):185-9.
39. Bayarri S, Gracia MJ, Lazaro R, Pe Rez-Arquillue C, Barberan M, Herrera A. Determination of the viability of *Toxoplasma gondii* in cured ham using bioassay: influence of technological processing and food safety implications. *Journal of food protection*. 2010;73(12):2239-43.
40. Ajzenberg D, Collinet F, Aubert D, Villena I, Darde ML, French ToxoBs network g, et al. The rural-urban effect on spatial genetic structure of type II *Toxoplasma gondii* strains involved in human congenital toxoplasmosis, France, 2002-2009. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2015;36:511-6.
41. Dubey JP, Desmonts G, McDonald C, Walls KW. Serologic evaluation of cattle inoculated with *Toxoplasma gondii*: comparison of Sabin-Feldman dye test and other agglutination tests. *American journal of veterinary research*. 1985;46(5):1085-8.
42. Desmonts G, Remington JS. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. *Journal of clinical microbiology*. 1980;11(6):562-8.

43. Sroka J, Karamon J, Cencek T, Dutkiewicz J. Preliminary assessment of usefulness of cELISA test for screening pig and cattle populations for presence of antibodies against *Toxoplasma gondii*. *Annals of agricultural and environmental medicine* : AAEM. 2011;18(2):335-9.
44. Gamble HR, Dubey JP, Lambillotte DN. Comparison of a commercial ELISA with the modified agglutination test for detection of *Toxoplasma* infection in the domestic pig. *Vet Parasitol*. 2005;128(3-4):177-81.
45. Steinparzer R, Reisp K, Grunberger B, Kofer J, Schmoll F, Sattler T. Comparison of different commercial serological tests for the detection of *Toxoplasma gondii* antibodies in serum of naturally exposed pigs. *Zoonoses Public Health*. 2015;62(2):119-24.
46. Klun I, Djurkovic-Djakovic O, Thulliez P. Comparison of a commercial ELISA with the modified agglutination test for the detection of *Toxoplasma gondii* infection in naturally exposed sheep. *Zoonoses Public Health*. 2007;54(3-4):165-8.
47. Halos L, Thebault A, Aubert D, Thomas M, Perret C, Geers R, et al. An innovative survey underlining the significant level of contamination by *Toxoplasma gondii* of ovine meat consumed in France. *Int J Parasitol*. 2010;40(2):193-200.
48. Hamidinejat H, Nabavi L, Mayahi M, Ghourbanpoor M, Pourmehdi Borojeni M, Norollahi Fard S, et al. Comparison of three diagnostic methods for the detection of *Toxoplasma gondii* in free range chickens. *Tropical biomedicine*. 2014;31(3):507-13.
49. Dubey JP, Laurin E, Kwok OC. Validation of the modified agglutination test for the detection of *Toxoplasma gondii* in free-range chickens by using cat and mouse bioassay. *Parasitology*. 2015:1-6.
50. Macri G, Sala M, Linder AM, Pettirossi N, Scarpulla M. Comparison of indirect fluorescent antibody test and modified agglutination test for detecting *Toxoplasma gondii* immunoglobulin G antibodies in dog and cat. *Parasitol Res*. 2009;105(1):35-40.
51. Santos TR, Costa AJ, Toniollo GH, Luvizotto MC, Benetti AH, Santos RR, et al. Prevalence of anti-*Toxoplasma gondii* antibodies in dairy cattle, dogs, and humans from the Jauru micro-region, Mato Grosso state, Brazil. *Vet Parasitol*. 2009;161(3-4):324-6.
52. Villena I, Chemla C, Quereux C, Dupouy D, Leroux B, Foudrinier F, et al. Prenatal diagnosis of congenital toxoplasmosis transmitted by an immunocompetent woman infected before conception. *Prenatal Diagnosis*. 1998;18:1079-81.
53. Thulliez P, Remington J, Santoro F, Ovlaque G, Sharma S, Desmonts G. Une nouvelle reaction d'agglutination pour le diagnostic du stade evolutif de la toxoplasmose acquise. *Pathologie Biologie*. 1986;34(4):173-7.
54. Villena I, Durand B, Aubert D, Blaga R, Geers R, Thomas M, et al. New strategy for the survey of *Toxoplasma gondii* in meat for human consumption. *Vet Parasitol*. 2012;183(3-4):203-8.
55. Auer H, Picher O, Aspöck H. Der Peroxidase test (ELISA) zum Nachweis von Antikörpern gegen *Toxoplasma gondii*. 1980 1980. Report No.
56. Bessieres-Cathala M. Specific and non-specific IgE: evaluation methods in toxoplasmosis using ELISA. *Lyon Medical*. 1982;248:85-8.
57. Picher O, Aspöck H. Die Bedeutung des Indirekten Haemagglutinationstests fuer die Diagnostik von *Toxoplasma*-Infektionen. 1980 1980. Report No.
58. Remington J. Detection and characterization of membrane antigens of *toxoplasma gondii*. *Lyon Medical*. 1982;248:9-13.
59. Remington J. A double-sandwich IgM ELISA for diagnosis of acute acquired and congenital *toxoplasma* infection. *Lyon Medical*. 1982;248:31-5.
60. Basso W, Hartnack S, Pardini L, Maksimov P, Koudela B, Venturini MC, et al. Assessment of diagnostic accuracy of a commercial ELISA for the detection of *Toxoplasma gondii* infection in pigs compared with IFAT, TgSAG1-ELISA and Western blot, using a Bayesian latent class approach. *Int J Parasitol*. 2013;43(7):565-70.
61. Bokken GC, Bergwerff AA, van Knapen F. A novel bead-based assay to detect specific antibody responses against *Toxoplasma gondii* and *Trichinella spiralis* simultaneously in sera of experimentally infected swine. *BMC Vet Res*. 2012;8:36.
62. Forbes LB, Parker SE, Gajadhar AA. Performance of commercial ELISA and agglutination test kits for the detection of anti-*Toxoplasma gondii* antibodies in serum and muscle fluid of swine infected with 100, 300, 500 or 1000 oocysts. *Vet Parasitol*. 2012;190(3-4):362-7.

63. Herrmann DC, Maksimov P, Maksimov A, Sutor A, Schwarz S, Jaschke W, et al. *Toxoplasma gondii* in foxes and rodents from the German Federal States of Brandenburg and Saxony-Anhalt: seroprevalence and genotypes. *Vet Parasitol.* 2012;185(2-4):78-85.
64. Jokelainen P, Isomursu M, Nareaho A, Oksanen A. Natural *Toxoplasma gondii* infections in European brown hares and mountain hares in Finland: proportional mortality rate, antibody prevalence, and genetic characterization. *Journal of wildlife diseases.* 2011;47(1):154-63.
65. Sobrino R, Cabezon O, Millan J, Pabon M, Arnal MC, Luco DF, et al. Seroprevalence of *Toxoplasma gondii* antibodies in wild carnivores from Spain. *Vet Parasitol.* 2007;148(3-4):187-92.
66. Burrells A, Bartley PM, Zimmer IA, Roy S, Kitchener AC, Meredith A, et al. Evidence of the three main clonal *Toxoplasma gondii* lineages from wild mammalian carnivores in the UK. *Parasitology.* 2013;140(14):1768-76.
67. Murphy TM, Walochnik J, Hassl A, Moriarty J, Mooney J, Toolan D, et al. Study on the prevalence of *Toxoplasma gondii* and *Neospora caninum* and molecular evidence of *Encephalitozoon cuniculi* and *Encephalitozoon (Septata) intestinalis* infections in red foxes (*Vulpes vulpes*) in rural Ireland. *Vet Parasitol.* 2007;146(3-4):227-34.
68. Aubert D, Ajzenberg D, Richomme C, Gilot-Fromont E, Terrier ME, de Gevigney C, et al. Molecular and biological characteristics of *Toxoplasma gondii* isolates from wildlife in France. *Vet Parasitol.* 2010;171(3-4):346-9.
69. Cabezon O, Resendes AR, Domingo M, Raga JA, Agusti C, Alegre F, et al. Seroprevalence of *Toxoplasma gondii* antibodies in wild dolphins from the Spanish Mediterranean coast. *The Journal of parasitology.* 2004;90(3):643-4.
70. Czopowicz M, Kaba J, Szalus-Jordanow O, Nowicki M, Witkowski L, Frymus T. Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infections in goats in Poland. *Vet Parasitol.* 2011;178(3-4):339-41.
71. Stimbirys A, Bagdonas J, Gerulis G, Russo P. A serological study on the prevalence of *Toxoplasma gondii* in sheep of Lithuania. *Polish journal of veterinary sciences.* 2007;10(2):83-7.
72. Jakubek EB, Lunden A, Ugglä A. Seroprevalences of *Toxoplasma gondii* and *Neospora* sp. infections in Swedish horses. *Vet Parasitol.* 2006;138(3-4):194-9.
73. Esteves F, Aguiar D, Rosado J, Costa ML, de Sousa B, Antunes F, et al. *Toxoplasma gondii* prevalence in cats from Lisbon and in pigs from centre and south of Portugal. *Vet Parasitol.* 2014;200(1-2):8-12.
74. Gauss CB, Dubey JP, Vidal D, Cabezon O, Ruiz-Fons F, Vicente J, et al. Prevalence of *Toxoplasma gondii* antibodies in red deer (*Cervus elaphus*) and other wild ruminants from Spain. *Vet Parasitol.* 2006;136(3-4):193-200.
75. Gyorko A, Opsteegh M, Mircean V, Iovu A, Cozma V. *Toxoplasma gondii* in Romanian household cats: evaluation of serological tests, epidemiology and risk factors. *Preventive veterinary medicine.* 2011;102(4):321-8.
76. Dorny P, Speybroeck N, Verstraete S, Baeke M, De Becker A, Berkvens D, et al. Serological survey of *Toxoplasma gondii*, feline immunodeficiency virus and feline leukaemia virus in urban stray cats in Belgium. *The Veterinary record.* 2002;151(21):626-9.
77. Opsteegh M, Teunis P, Mensink M, Zuchner L, Titilincu A, Langelaar M, et al. Evaluation of ELISA test characteristics and estimation of *Toxoplasma gondii* seroprevalence in Dutch sheep using mixture models. *Preventive veterinary medicine.* 2010;96(3-4):232-40.
78. Klun I, Djurkovic-Djakovic O, Katic-Radivojevic S, Nikolic A. Cross-sectional survey on *Toxoplasma gondii* infection in cattle, sheep and pigs in Serbia: seroprevalence and risk factors. *Vet Parasitol.* 2006;135(2):121-31.
79. Lopes AP, Dubey JP, Neto F, Rodrigues A, Martins T, Rodrigues M, et al. Seroprevalence of *Toxoplasma gondii* infection in cattle, sheep, goats and pigs from the North of Portugal for human consumption. *Vet Parasitol.* 2013;193(1-3):266-9.
80. Garcia-Bocanegra I, Cabezon O, Hernandez E, Martinez-Cruz MS, Martinez-Moreno A, Martinez-Moreno J. *Toxoplasma gondii* in ruminant species (cattle, sheep, and goats) from southern Spain. *The Journal of parasitology.* 2013;99(3):438-40.
81. Gilot-Fromont E, Aubert D, Belkilani S, Hermitte P, Gibout O, Geers R, et al. Landscape, herd management and within-herd seroprevalence of *Toxoplasma gondii* in beef cattle herds from Champagne-Ardenne, France. *Vet Parasitol.* 2009;161(1-2):36-40.

82. Maksimov P, Buschtöns S, Herrmann DC, Conraths FJ, Gorlich K, Tenter AM, et al. Serological survey and risk factors for *Toxoplasma gondii* in domestic ducks and geese in Lower Saxony, Germany. *Vet Parasitol.* 2011;182(2-4):140-9.
83. Koethe M, Pott S, Ludewig M, Bangoura B, Zoller B, Dauschies A, et al. Prevalence of specific IgG-antibodies against *Toxoplasma gondii* in domestic turkeys determined by kinetic ELISA based on recombinant GRA7 and GRA8. *Vet Parasitol.* 2011;180(3-4):179-90.
84. Dubey JP, Edelhofer R, Marcet P, Vianna MC, Kwok OC, Lehmann T. Genetic and biologic characteristics of *Toxoplasma gondii* infections in free-range chickens from Austria. *Vet Parasitol.* 2005;133(4):299-306.
85. Dubey JP, Vianna MC, Sousa S, Canada N, Meireles S, Correia da Costa JM, et al. Characterization of *Toxoplasma gondii* isolates in free-range chickens from Portugal. *The Journal of parasitology.* 2006;92(1):184-6.
86. Garcia-Bocanegra I, Simon-Grife M, Dubey JP, Casal J, Martin GE, Cabezon O, et al. Seroprevalence and risk factors associated with *Toxoplasma gondii* in domestic pigs from Spain. *Parasitology international.* 2010;59(3):421-6.
87. van der Giessen JF, M.; Bouwknegt, M.; Langelaar, M.; Vollema, A. Seroprevalence of *Trichinella spiralis* and *Toxoplasma gondii* in pigs from different housing systems in The Netherlands. *Vet Parasitol.* 2007;148(3-4):371-4.
88. Berger-Schoch AE, Bernet D, Doherr MG, Gottstein B, Frey CF. *Toxoplasma gondii* in Switzerland: a serosurvey based on meat juice analysis of slaughtered pigs, wild boar, sheep and cattle. *Zoonoses Public Health.* 2011;58(7):472-8.
89. Turcekova LA, D.; Reiterova, K.; Spisak, F. Occurrence and genetic characterization of *Toxoplasma gondii* in naturally infected pigs. *Acta parasitologica / Witold Stefanski Institute of Parasitology, Warszawa, Poland.* 2013;58(3):361-6.
90. Pastiu AIG, A.; Blaga, R.; Mircean, V.; Rosenthal, B. M.; Cozma, V. In Romania, exposure to *Toxoplasma gondii* occurs twice as often in swine raised for familial consumption as in hunted wild boar, but occurs rarely, if ever, among fattening pigs raised in confinement. *Parasitol Res.* 2013;112(6):2403-7.
91. Jurankova J, Basso W, Neumayerova H, Balaz V, Janova E, Sidler X, et al. Brain is the predilection site of *Toxoplasma gondii* in experimentally inoculated pigs as revealed by magnetic capture and real-time PCR. *Food microbiology.* 2014;38:167-70.
92. Hughes H, Kasper L, Little J, Dubey J. Absence of a role for NK cells in the control of acute infection by *T.gondii* oocysts. *Clinical Experimental Immunology.* 1978;72:394-9.
93. Djurkovic-Djakovic O, Djokic V, Vujanic M, Zivkovic T, Bobic B, Nikolic A, et al. Kinetics of parasite burdens in blood and tissues during murine toxoplasmosis. *Experimental parasitology.* 2012;131(3):372-6.
94. Dubey JP. Infectivity and pathogenicity of *Toxoplasma gondii* oocysts for cats. *The Journal of parasitology.* 1996;82(6):957-61.
95. Dubey JP, Hill DE, Jones JL, Hightower AW, Kirkland E, Roberts JM, et al. Prevalence of viable *Toxoplasma gondii* in beef, chicken, and pork from retail meat stores in the United States: risk assessment to consumers. *The Journal of parasitology.* 2005;91(5):1082-93.
96. Grover C, Thulliez P, Remington J, Boothroyd J. Rapid prenatal diagnosis of congenital *Toxoplasma* infection by using polymerase chain reaction and amniotic fluid. *Journal of clinical microbiology.* 1990;28(No.10):2297-301.
97. Homan W, Vercammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *International Journal for Parasitology.* 2000;30(1):69-75.
98. Grigg ME, Boothroyd JC. Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the B1 gene. *Journal of clinical microbiology.* 2001;39(1):398-400.
99. Mancianti F, Nardoni S, Mugnaini L, Poli A. *Toxoplasma gondii* in waterfowl: the first detection of this parasite in *Anas crecca* and *Anas clypeata* from Italy. *The Journal of parasitology.* 2013;99(3):561-3.

100. Hughes JM, Thomasson D, Craig PS, Georgin S, Pickles A, Hide G. *Neospora caninum*: detection in wild rabbits and investigation of co-infection with *Toxoplasma gondii* by PCR analysis. *Experimental parasitology*. 2008;120(3):255-60.
101. Nogareda F, Le Strat Y, Villena I, De Valk H, Goulet V. Incidence and prevalence of *Toxoplasma gondii* infection in women in France, 1980-2020: model-based estimation. *Epidemiology and infection*. 2014;142(8):1661-70.
102. Hill DE, Dubey JP. *Toxoplasma gondii* prevalence in farm animals in the United States. *Int J Parasitol*. 2013;43(2):107-13.
103. Hill DE, Benedetto SM, Coss C, McCrary JL, Fournet VM, Dubey JP. Effects of time and temperature on the viability of *Toxoplasma gondii* tissue cysts in enhanced pork loin. *Journal of food protection*. 2006;69(8):1961-5.
104. Dubey JP, Kotula AW, Sharar A, Andrews CD, Lindsay DS. Effect of high temperature on infectivity of *Toxoplasma gondii* tissue cysts in pork. *The Journal of parasitology*. 1990;76(2):201-4.
105. Lindsay DS, Collins MV, Holliman D, Flick GJ, Dubey JP. Effects of high-pressure processing on *Toxoplasma gondii* tissue cysts in ground pork. *The Journal of parasitology*. 2006;92(1):195-6.
106. Neumayerova H, Jurankova J, Salakova A, Gallas L, Kovarcik K, Koudela B. Survival of experimentally induced *Toxoplasma gondii* tissue cysts in vacuum packed goat meat and dry fermented goat meat sausages. *Food microbiology*. 2014;39:47-52.
107. Hill DE, Sreekumar C, Gamble HR, Dubey JP. Effect of commonly used enhancement solutions on the viability of *Toxoplasma gondii* tissue cysts in pork loin. *Journal of food protection*. 2004;67(10):2230-3.
108. Djokic V, Blaga R, Aubert D, Durand B, Perret C, Geers R, et al. *Toxoplasma gondii* infection in pork produced in France. *Parasitology*. 2016.
109. Fablet C, Dorenlor V, Eono F, Eveno E, Jolly JP, Portier F, et al. Noninfectious factors associated with pneumonia and pleuritis in slaughtered pigs from 143 farrow-to-finish pig farms. *Preventive veterinary medicine*. 2012;104(3-4):271-80.
110. Agreste. Les élevages de porcs en France métropolitaine en 2010. Agreste Primeur. 2013;300.
111. Cabezon O, Garcia-Bocanegra I, Molina-Lopez R, Marco I, Blanco JM, Hofle U, et al. Seropositivity and risk factors associated with *Toxoplasma gondii* infection in wild birds from Spain. *PLoS One*. 2011;6(12):e29549.
112. Pardini L, Maksimov P, Herrmann DC, Bacigalupe D, Rambeaud M, Machuca M, et al. Evaluation of an in-house TgSAG1 (P30) IgG ELISA for diagnosis of naturally acquired *Toxoplasma gondii* infection in pigs. *Vet Parasitol*. 2012;189(2-4):204-10.
113. R development Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing; 2010.
114. Venables WN, Ripley BD. *Modern Applied Statistics with S*. New York: Springer; 2002.
115. Bates D, Maechler M, Bolker BM, Walker S. *Lme4: Linear mixed-effects models using Eigen and S4*. 2014.
116. Fox J, Weisberg S. *An (R) Copanion to Applied Regression*. second ed. Thousand Oaks, CA: Sage; 2011.
117. Garcia-Bocanegra I, Dubey JP, Simon-Grife M, Cabezon O, Casal J, Allepuz A, et al. Seroprevalence and risk factors associated with *Toxoplasma gondii* infection in pig farms from Catalonia, north-eastern Spain. *Res Vet Sci*. 2010;89(1):85-7.
118. Damriyasa IM, Bauer C, Edelhofer R, Failing K, Lind P, Petersen E, et al. Cross-sectional survey in pig breeding farms in Hesse, Germany: seroprevalence and risk factors of infections with *Toxoplasma gondii*, *Sarcocystis* spp. and *Neospora caninum* in sows. *Vet Parasitol*. 2004;126(3):271-86.
119. Dekšne G, Kirjusina M. Seroprevalence of *Toxoplasma gondii* in domestic pigs (*Sus scrofa domestica*) and wild boars (*Sus scrofa*) in Latvia. *The Journal of parasitology*. 2013;99(1):44-7.
120. Klun IV, M.; Yera, H.; Nikolic, A.; Iovic, V.; Bobic, B.; Bradonjic, S.; Dupouy-Camet, J.; Djurkovic-Djakovic, O. *Toxoplasma gondii* infection in slaughter pigs in Serbia: seroprevalence and demonstration of parasites in blood. *Vet Res*. 2011;42(1):17.

121. Veronesi F, Ranucci D, Branciarri R, Miraglia D, Mammoli R, Fioretti DP. Seroprevalence and risk factors for *Toxoplasma gondii* infection on finishing swine reared in the Umbria region, central Italy. *Zoonoses Public Health*. 2011;58(3):178-84.
122. Lopes WD, Santos TR, Luvizotto MC, Sakamoto CA, Oliveira GP, Costa AJ. Histopathology of the reproductive system of male sheep experimentally infected with *Toxoplasma gondii*. *Parasitol Res*. 2011;109(2):405-9.
123. Garcia-Bocanegra I, Simon-Grife M, Sibila M, Dubey JP, Cabezon O, Martin G, et al. Duration of maternally derived antibodies in *Toxoplasma gondii* naturally infected piglets. *Vet Parasitol*. 2010;170(1-2):134-6.
124. Dubey JP, Urban JF, Jr. Diagnosis of transplacentally induced toxoplasmosis in pigs. *American journal of veterinary research*. 1990;51(8):1295-9.
125. Lind P, Haugegaard J, Wingstrand A, Henriksen SA. The time course of the specific antibody response by various ELISAs in pigs experimentally infected with *Toxoplasma gondii*. *Vet Parasitol*. 1997;71(1):1-15.
126. Fablet C, Marois-Crehan C, Simon G, Grasland B, Jestin A, Kobisch M, et al. Infectious agents associated with respiratory diseases in 125 farrow-to-finish pig herds: a cross-sectional study. *Veterinary microbiology*. 2012;157(1-2):152-63.
127. Damriyasa IM, Bauer C. Seroprevalence of *Toxoplasma gondii* infection in sows in Munsterland, Germany. *DTW Deutsche tierärztliche Wochenschrift*. 2005;112(6):223-4.
128. Lunden A, Lind P, Engvall EO, Gustavsson K, Uggla A, Vagsholm I. Serological survey of *Toxoplasma gondii* infection in pigs slaughtered in Sweden. *Scandinavian journal of infectious diseases*. 2002;34(5):362-5.
129. de Sousa SA, D.; Canada, N.; Freire, L.; da Costa, J. M.; Darde, M. L.; Thulliez, P.; Dubey, J. P. Biologic and molecular characterization of *Toxoplasma gondii* isolates from pigs from Portugal. *Vet Parasitol*. 2006;135(2):133-6.
130. Basso W, Handke M, Sydler T, Borel N, Grimm F, Sidler X, et al. Involvement of *Toxoplasma gondii* in reproductive disorders in Swiss pig farms. *Parasitology international*. 2015;64(2):157-60.
131. Wallander C, Frossling J, Vagsholm I, Uggla A, Lunden A. *Toxoplasma gondii* seroprevalence in wild boars (*Sus scrofa*) in Sweden and evaluation of ELISA test performance. *Epidemiology and infection*. 2015;143(9):1913-21.
132. Racka K, Bartova E, Budikova M, Vodrazka P. Survey of *Toxoplasma gondii* antibodies in meat juice of wild boar (*Sus scrofa*) in several districts of the Czech Republic. *Annals of agricultural and environmental medicine : AAEM*. 2015;22(2):231-5.
133. Antolova D, Reiterova K, Dubinsky P. Seroprevalence of *Toxoplasma gondii* in wild boars (*Sus scrofa*) in the Slovak Republic. *Annals of agricultural and environmental medicine : AAEM*. 2007;14(1):71-3.
134. Touloudi A, Valiakos G, Athanasiou LV, Birtsas P, Giannakopoulos A, Papaspyropoulos K, et al. A serosurvey for selected pathogens in Greek European wild boar. *Vet Rec Open*. 2015;2(2):e000077.
135. Gauss CB, Dubey JP, Vidal D, Ruiz F, Vicente J, Marco I, et al. Seroprevalence of *Toxoplasma gondii* in wild pigs (*Sus scrofa*) from Spain. *Vet Parasitol*. 2005;131(1-2):151-6.
136. Coelho C, Vieira-Pinto M, Faria AS, Vale-Goncalves H, Veloso O, Paiva-Cardoso M, et al. Serological evidence of *Toxoplasma gondii* in hunted wild boar from Portugal. *Vet Parasitol*. 2014;202(3-4):310-2.
137. Richomme C, Afonso E, Tolon V, Ducrot C, Halos L, Alliot A, et al. Seroprevalence and factors associated with *Toxoplasma gondii* infection in wild boar (*Sus scrofa*) in a Mediterranean island. *Epidemiology and infection*. 2010;138(9):1257-66.
138. Beral M, Rossi S, Aubert D, Gasqui P, Terrier ME, Klein F, et al. Environmental factors associated with the seroprevalence of *Toxoplasma gondii* in Wild Boars (*Sus scrofa*), France. *EcoHealth*. 2012;9(3):303-9.
139. Witkowski L, Czopowicz M, Nagy DA, Potarniche AV, Aoanei MA, Imomov N, et al. Seroprevalence of *Toxoplasma gondii* in wild boars, red deer and roe deer in Poland. *Parasite*. 2015;22:17.

140. Bayarri S, Gracia MJ, Perez-Arquillue C, Lazaro R, Herrera A. Toxoplasma gondii in commercially available pork meat and cured ham: a contribution to risk assessment for consumers. Journal of food protection. 2012;75(3):597-600.
141. Batz MB, Hoffmann S, Morris JG, Jr. 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. 2012;75(7):1278-91.
142. Chang QC, Zheng X, Qiu JH, Wang CR, Zhu XQ. Seroprevalence of Toxoplasma gondii infection in fattening pigs in Northeast China. The Journal of parasitology. 2013;99(3):544-5.
143. Chandrawathani P, Nurulaini R, Zanin CM, Premaalatha B, Adnan M, Jamnah O, et al. Seroprevalence of Toxoplasma gondii antibodies in pigs, goats, cattle, dogs and cats in peninsular Malaysia. Tropical biomedicine. 2008;25(3):257-8.
144. Pezerico SB, Langoni H, Da Silva AV, Da Silva RC. Evaluation of Toxoplasma gondii placental transmission in BALB/c mice model. Experimental parasitology. 2009;123(2):168-72.
145. Guo M, Dubey JP, Hill D, Buchanan RL, Gamble HR, Jones JL, et al. Prevalence and risk factors for Toxoplasma gondii infection in meat animals and meat products destined for human consumption. Journal of food protection. 2015;78(2):457-76.
146. Feitosa TFV, V. L.; de Melo, L. R.; de Almeida Neto, J. L.; Souto, D. V.; de Moraes, D. F.; Athayde, A. C.; Azevedo, S. S.; Pena, H. F. Toxoplasma gondii and Neospora caninum in slaughtered pigs from Northeast, Brazil. Vet Parasitol. 2014;202(3-4):305-9.
147. Samico Fernandes EF, Samico Fernandes MF, Kim PC, de Albuquerque PP, de Souza Neto OL, de SSA, et al. Prevalence of Toxoplasma gondii in slaughtered pigs in the state of Pernambuco, Brazil. The Journal of parasitology. 2012;98(3):690-1.
148. dos Santos CB, de Carvalho AC, Ragozo AM, Soares RM, Amaku M, Yai LE, et al. First isolation and molecular characterization of Toxoplasma gondii from finishing pigs from Sao Paulo State, Brazil. Vet Parasitol. 2005;131(3-4):207-11.
149. Cademartori BG, Santos LM, Oliveira FC, Quevedo P, Oliveira PA, Ramos TS, et al. Isolation and pathogenicity of Toxoplasma gondii in naturally infected (rustic farm) pigs in southern Brazil. Vet Parasitol. 2014;203(1-2):207-11.
150. Wyss R, Sager H, Muller N, Inderbitzin F, Konig M, Audige L, et al. [The occurrence of Toxoplasma gondii and Neospora caninum as regards meat hygiene]. Schweizer Archiv fur Tierheilkunde. 2000;142(3):95-108.
151. Frazao-Teixeira ES, N.; Dubey, J. P.; Grigg, M. E.; de Oliveira, F. C. Multi-locus DNA sequencing of Toxoplasma gondii isolated from Brazilian pigs identifies genetically divergent strains. Vet Parasitol. 2011;175(1-2):33-9.
152. Galvan-Ramirez ML, Madriz Elisondo AL, Rico Torres CP, Luna-Pasten H, Rodriguez Perez LR, Rincon-Sanchez AR, et al. Frequency of Toxoplasma gondii in pork meat in Ocotlan, Jalisco, Mexico. Journal of food protection. 2010;73(6):1121-3.
153. Velmurugan GVS, C.; Dubey, J. P. Isolate designation and characterization of Toxoplasma gondii isolates from pigs in the United States. The Journal of parasitology. 2009;95(1):95-9.
154. Dubey JP, Hill DE, Sundar N, Velmurugan GV, Bandini LA, Kwok OC, et al. Endemic toxoplasmosis in pigs on a farm in Maryland: isolation and genetic characterization of Toxoplasma gondii. The Journal of parasitology. 2008;94(1):36-41.
155. da Silva AV, Pezerico SB, de Lima VY, d'Arc Moretti L, Pinheiro JP, Tanaka EM, et al. Genotyping of Toxoplasma gondii strains isolated from dogs with neurological signs. Vet Parasitol. 2005;127(1):23-7.
156. Dubey JP, Andrews CD, Lind P, Kwok OC, Thulliez P, Lunney JK. Antibody responses measured by various serologic tests in pigs orally inoculated with low numbers of Toxoplasma gondii oocysts. American journal of veterinary research. 1996;57(12):1733-7.
157. Opsteegh M, Langelaar M, Sprong H, den Hartog L, De Craeye S, Bokken G, et al. Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR. International journal of food microbiology. 2010;139(3):193-201.
158. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977;33(1):159-74.
159. Nakazawa N. fmsb: Functions for Medical Statistics Book with some Demographic Data. 2015.

435 160. Kuhn M. caret: Classification and Regression Training. 2015.

436 161. Terkawi MA, Kameyama K, Rasul NH, Xuan X, Nishikawa Y. Development of an
437 immunochromatographic assay based on dense granule protein 7 for serological detection of
438 *Toxoplasma gondii* infection. Clinical and vaccine immunology : CVI. 2013;20(4):596-601.

439 162. Kapel CM, Webster P, Lind P, Pozio E, Henriksen SA, Murrell KD, et al. *Trichinella spiralis*,
440 *T. britovi*, and *T. nativa*: infectivity, larval distribution in muscle, and antibody response after
441 experimental infection of pigs. Parasitol Res. 1998;84(4):264-71.

442 163. Gajadhar AA, Pozio E, Gamble HR, Nockler K, Maddox-Hyttel C, Forbes LB, et al.
443 *Trichinella* diagnostics and control: mandatory and best practices for ensuring food safety. Vet
444 Parasitol. 2009;159(3-4):197-205.

445 164. Hezard N, Marx-Chemla C, Foudrinier F, Villena I, Quereux C, Leroux B, et al. Prenatal
446 diagnosis of congenital toxoplasmosis in 261 pregnancies. Prenat Diagn. 1997;17(11):1047-54.

447 165. Fricker-Hidalgo H, Cimon B, Chemla C, Darde ML, Delhaes L, L'Ollivier C, et al.
448 *Toxoplasma* seroconversion with negative or transient immunoglobulin M in pregnant women: myth
449 or reality? A French multicenter retrospective study. Journal of clinical microbiology.
450 2013;51(7):2103-11.

451 166. Cavalcante GT, Aguilar DM, Camargo LM, Labruna MB, de Andrade HF, Meireles LR, et al.
452 Seroprevalence of *Toxoplasma gondii* antibodies in humans from rural Western Amazon, Brazil. The
453 Journal of parasitology. 2006;92(3):647-9.

454 167. Dubey JP, Andrews CD, Thulliez P, Lind P, Kwok OC. Long-term humoral antibody
455 responses by various serologic tests in pigs orally inoculated with oocysts of four strains of
456 *Toxoplasma gondii*. Vet Parasitol. 1997;68(1-2):41-50.

457 168. Dubey JP, Rollor EA, Smith K, Kwok OC, Thulliez P. Low seroprevalence of *Toxoplasma*
458 *gondii* in feral pigs from a remote island lacking cats. The Journal of parasitology. 1997;83(5):839-41.

459 169. Cosendey-KezenLeite RI, de Oliveira FC, Frazao-Teixeira E, Dubey JP, de Souza GN,
460 Ferreira AM, et al. Occurrence and risk factors associated to *Toxoplasma gondii* infection in sheep
461 from Rio de Janeiro, Brazil. Trop Anim Health Prod. 2014;46(8):1463-6.

462 170. Cai Y, Wang Z, Li J, Li N, Wei F, Liu Q. Evaluation of an indirect ELISA using recombinant
463 granule antigen Gra7 for serodiagnosis of *Toxoplasma gondii* infection in cats. The Journal of
464 parasitology. 2015;101(1):37-40.

465 171. Lopes AP, Granada S, Oliveira AC, Brancal H, Dubey JP, Cardoso L, et al. Toxoplasmosis in
466 dogs: first report of *Toxoplasma gondii* infection in any animal species in Angola. Pathogens and
467 global health. 2014;108(7):344-6.

468 172. Tavalla M, Sabaghan M, Abdizadeh R, Khademvatan S, Rafiei A, Razavi Piranshahi A.
469 Seroprevalence of *Toxoplasma gondii* and *Neospora* spp. Infections in Arab Horses, Southwest of
470 Iran. Jundishapur journal of microbiology. 2015;8(3):e14939.

471 173. Alvarado-Esquivel C, Vazquez-Morales RF, Colado-Romero EE, Guzman-Sanchez R,
472 Liesenfeld O, Dubey JP. Prevalence of infection with *Toxoplasma gondii* in landrace and mixed breed
473 pigs slaughtered in Baja California Sur State, Mexico. European journal of microbiology &
474 immunology. 2015;5(1):112-5.

475 174. Yin MY, Wang JL, Huang SY, Qin SY, Zhou DH, Liu GX, et al. Seroprevalence and risk
476 factors of *Toxoplasma gondii* in Tibetan Sheep in Gansu province, Northwestern China. BMC Vet
477 Res. 2015;11(1):41.

478 175. Gennari SM, Ogrzewalska MH, Soares HS, Saraiva DG, Pinter A, Nieri-Bastos FA, et al.
479 *Toxoplasma gondii* antibodies in wild rodents and marsupials from the Atlantic Forest, state of Sao
480 Paulo, Brazil. Rev Bras Parasitol Vet. 2015;24(3):379-82.

481 176. Tenter AM. *Toxoplasma gondii* in animals used for human consumption. Mem Inst Oswaldo
482 Cruz. 2009;104(2):364-9.

483 177. Wallander C, Frossling J, Vagsholm I, Burrells A, Lunden A. "Meat juice" is not a
484 homogeneous serological matrix. Foodborne pathogens and disease. 2015;12(4):280-8.

485 178. Wingstrand A, Lind P, Haugegaard J, Henriksen SA, Bille-Hansen V, Sorensen V. Clinical
486 observations, pathology, bioassay in mice and serological response at slaughter in pigs experimentally
487 infected with *Toxoplasma gondii*. Vet Parasitol. 1997;72(2):129-40.

488 179. Dubey JP, Lunney JK, Shen SK, Kwok OC. Immunity to toxoplasmosis in pigs fed irradiated
489 *Toxoplasma gondii* oocysts. The Journal of parasitology. 1998;84(4):749-52.

180. Dubey JP, Lunney JK, Shen SK, Kwok OC, Ashford DA, Thulliez P. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. *The Journal of parasitology*. 1996;82(3):438-43.
181. Dubey JP. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. *American journal of veterinary research*. 1988;49(6):910-3.
182. Kotula AW, Dubey JP, Sharar AK, Andrews CD, Shen SK, Lindsay DS. Effect of Freezing on Infectivity of *Toxoplasma Gondii* Tissue Cysts in Pork. *Journal of food protection*. 1991;54(9):687-90.
183. Delhaes L, Filisetti D, Brenier-Pinchart MP, Pelloux H, Yera H, Dalle F, et al. Freezing and storage at -20 degrees C provides adequate preservation of *Toxoplasma gondii* DNA for retrospective molecular analysis. *Diagn Microbiol Infect Dis*. 2014;80(3):197-9.
184. Belleste B, Flori P, Hafid J, Raberin H, Tran Manh Sung R. Influence of the quantity of nonspecific DNA and repeated freezing and thawing of samples on the quantification of DNA by the Light Cycler. *Journal of microbiological methods*. 2003;55(1):213-9.
185. Cano-Munoz G. Manual on meat cold store operation and management. FAO ANIMAL PRODUCTION AND HEALTH PAPER [Internet]. 1991; 92. Available from: <http://www.fao.org/docrep/004/t0098e/T0098E00.htm#TOC>.
186. Garcia JL, Gennari SM, Machado RZ, Navarro IT. *Toxoplasma gondii*: detection by mouse bioassay, histopathology, and polymerase chain reaction in tissues from experimentally infected pigs. *Experimental parasitology*. 2006;113(4):267-71.
187. Dubey JP. Refinement of pepsin digestion method for isolation of *Toxoplasma gondii* from infected tissues. *Vet Parasitol*. 1998;74(1):75-7.
188. Dubey J. Re-examination of resistance of *Toxoplasma gondii* tachyzoites and bradyzoites to pepsin and trypsin digestion. *Parasitology*. 1998;116:43-50.
189. da Silva AV, Langoni H. The detection of *Toxoplasma gondii* by comparing cytology, histopathology, bioassay in mice, and the polymerase chain reaction (PCR). *Vet Parasitol*. 2001;97(3):191-8.
190. Dubey JP. Strategies to reduce transmission of *Toxoplasma gondii* to animals and humans. *Vet Parasitol*. 1996;64(1-2):65-70.
191. Hill D, Coss C, Dubey JP, Wroblewski K, Sautter M, Hosten T, et al. Identification of a sporozoite-specific antigen from *Toxoplasma gondii*. *The Journal of parasitology*. 2011;97(2):328-37.
192. Torrey EF, Yolken RH. *Toxoplasma* oocysts as a public health problem. *Trends in parasitology*. 2013;29(8):380-4.
193. Sullivan WJ, Jr., Jeffers V. Mechanisms of *Toxoplasma gondii* persistence and latency. *FEMS Microbiol Rev*. 2012;36(3):717-33.
194. Holz J, Albrecht M. [Culture of *Toxoplasma gondii* in cell cultures]. *Zeitschrift fur Hygiene und Infektionskrankheiten; medizinische Mikrobiologie, Immunologie und Virologie*. 1953;136(6):605-9.
195. Ashburn D, Evans R, Chatterton JM, Joss AW, Ho-Yen DO. *Toxoplasma* dye test using cell culture derived tachyzoites. *J Clin Pathol*. 2000;53(8):630-3.
196. Mavin S, Joss A, Ball J, Ho-Yen D. Do *Toxoplasma gondii* RH strain tachyzoites evolve during continuous passage? *Journal of Clinical Pathology*. 2004;57:609-11.
197. Hassl A, Aspöck H. A rapid and simple method of purification of *Toxoplasma gondii* trophozoites originating from tissue culture for use in the indirect immunofluorescent antibody test. *Zentralblatt für Bakteriologie : international journal of medical microbiology*. 1990;272(4):509-13.
198. Suresh K, Mak JW, Yong HS. Long term maintenance of *Toxoplasma gondii* (Rh strain) in Vero cell line and use of harvested antigens for immunodiagnosis. *The Southeast Asian journal of tropical medicine and public health*. 1991;22 Suppl:124-8.
199. Hayes K, Billson FA, Jack I, Sanderson L, Rogers JG, Greer CH, et al. Cell culture isolation of *Toxoplasma gondii* from an infant with unusual ocular features. *Med J Aust*. 1973;1(26):1297-9.
200. Kumar PS, Mohapatra LN, Balaya S, Kumar R. Haemagglutination antigen from *Toxoplasma gondii* grown in tissue culture. *The Indian journal of medical research*. 1977;66(5):756-64.
201. Davoust B, Mediannikov O, Sambou M, Roqueplo C, Demoncheaux JP, Perret C, et al. [Serological survey of animal toxoplasmosis in Senegal.]. *Bull Soc Pathol Exot*. 2014.
202. Hamilton CM, Kelly PJ, Bartley PM, Burrells A, Porco A, Metzler D, et al. *Toxoplasma gondii* in livestock in St. Kitts and Nevis, West Indies. *Parasit Vectors*. 2015;8(1):166.

545 203. Hitt JA, Filice GA. Detection of *Toxoplasma gondii* parasitemia by gene amplification, cell
546 culture, and mouse inoculation. *Journal of clinical microbiology*. 1992;30(12):3181-4.

547 204. James GS, Sintchenko VG, Dickeson DJ, Gilbert GL. Comparison of cell culture, mouse
548 inoculation, and PCR for detection of *Toxoplasma gondii*: effects of storage conditions on sensitivity.
549 *Journal of clinical microbiology*. 1996;34(6):1572-5.

550 205. Lavrard I, Chouaid C, Roux P, Poirot JL, Marteau M, Lemarchand B, et al. Pulmonary
551 toxoplasmosis in HIV-infected patients: usefulness of polymerase chain reaction and cell culture. *The*
552 *European respiratory journal*. 1995;8(5):697-700.

553 206. Wu L, Zhang QX, Li TT, Chen SX, Cao JP. [In vitro culture of *Toxoplasma gondii*
554 tachyzoites in HFF and HeLa cells]. *Zhongguo ji sheng chong xue yu ji sheng chong bing za zhi* =
555 *Chinese journal of parasitology & parasitic diseases*. 2009;27(3):229-31.

556 207. Soete M, Camus D, Dubremetz JF. Experimental induction of bradyzoite-specific antigen
557 expression and cyst formation by the RH strain of *Toxoplasma gondii* in vitro. *Experimental*
558 *parasitology*. 1994;78(4):361-70.

559 208. Akinshina GT. [the Use of Different Cell Types for Cultivating *Toxoplasma Gondii*].
560 *Biulleten' eksperimental'noi biologii i meditsiny*. 1963;56:69-72.

561 209. Hansson HA, Sourander P. *Toxoplasma Gondii* in Cell Cultures from Rat Retina. *Virchows*
562 *Archiv fur pathologische Anatomie und Physiologie und fur klinische Medizin*. 1965;338:224-36.

563 210. Zuckerman A. Propagation of parasitic protozoa in tissue culture and avian embryos. *Ann N Y*
564 *Acad Sci*. 1966;139(1):24-38.

565 211. Akinshina GT, Zasukhin DN, Bliumkin VN, Levina DS, Gavrilov VI. [Cultivation of
566 *toxoplasma* in transplantable swine embryo kidney cell cultures (RES and RES-La)]. *Biulleten'*
567 *eksperimental'noi biologii i meditsiny*. 1967;64(9):69-71.

568 212. Franchi P, Hahn EE. *Toxoplasma gondii* in tissue culture and maintenance at low
569 temperatures. *Zeitschrift fur Parasitenkunde (Berlin, Germany)*. 1968;30(4):360-7.

570 213. Csoka R, Kulcsar G. Maintenance of *Toxoplasma gondii* in primary human amniotic cell
571 culture. *Acta microbiologica Academiae Scientiarum Hungaricae*. 1968;15(4):357-60.

572 214. Bommer W, Hofling KH, Heunert HH. [Multiplication of *toxoplasma gondii* in cell cultures].
573 *German medical monthly*. 1969;14(8):399-405.

574 215. Bollinger RO, Musallam N, Stulberg CS. Freeze preservation of tissue culture propagated
575 *Toxoplasma gondii*. *The Journal of parasitology*. 1974;60(2):368-9.

576 216. Remington JS, Earle P, Yagura T. *Toxoplasma* in nucleus. *The Journal of parasitology*.
577 1970;56(2):390-1.

578 217. Sheffield HG, Melton ML. *Toxoplasma gondii*: the oocyst, sporozoite, and infection of
579 cultured cells. *Science*. 1970;167(3919):892-3.

580 218. Krahenbuhl JL, Blazkovec AA, Lysenko MG. The use of tissue culture-grown trophozoites of
581 an avirulent strain of *Toxoplasma gondii* for the immunization of mice and guinea pigs. *The Journal of*
582 *parasitology*. 1971;57(2):386-90.

583 219. Buckley SM. Survival of *Toxoplasma gondii* in mosquito cell lines and establishment of
584 continuous infection in Vero cell cultures. *Experimental parasitology*. 1973;33(1):23-6.

585 220. Anderson SE, Jr., Remington JS. Effect of normal and activated human macrophages on
586 *Toxoplasma gondii*. *J Exp Med*. 1974;139(5):1154-74.

587 221. Rose NR, Milisauskas V, Zeff G. Antigenic and enzymatic changes in infected and
588 transformed human diploid cells. *Immunol Commun*. 1975;4(1):1-16.

589 222. Braveny I, Winter W, Disko R. A method of mass cultivation of *Toxoplasma gondii* in cell
590 culture. *Tropenmed Parasitol*. 1978;29(4):432-4.

591 223. Grimwood BG, Hechemy K, Stevens RW. *Toxoplasma gondii*: purification of trophozoites
592 propagated in cell culture. *Experimental parasitology*. 1979;48(2):282-6.

593 224. Gupta SL, Gautam OP, Bhardwaj RM. Cultivation of *Toxoplasma gondii* in lamb testicular
594 cell culture. *The Indian journal of medical research*. 1980;71:217-20.

595 225. Werk R, Fischer S. Attempts to infect plant protoplasts with *Toxoplasma gondii*. *Journal of*
596 *general microbiology*. 1982;128(1):211-3.

597 226. Hofflin JM, Remington JS. Tissue culture isolation of *Toxoplasma* from blood of a patient
598 with AIDS. *Arch Intern Med*. 1985;145(5):925-6.

227. Lindsay D, Dubey J, Blagburn B, Toivio-Kinnucan M. Examination of tissue cyst formation by *Toxoplasma gondii* in cell cultures using bradyzoites, tachyzoites and sporozoites. *The Journal of parasitology*. 1991;77(1):126-32.
228. Soete M, Fortier B, Camus D, Dubremetz JF. *Toxoplasma gondii*: kinetics of bradyzoite-tachyzoite interconversion in vitro. *Experimental parasitology*. 1993;76(3):259-64.
229. Weiss LM, Laplace D, Takvorian PM, Tanowitz HB, Cali A, Wittner M. A cell culture system for study of the development of *Toxoplasma gondii* bradyzoites. *The Journal of eukaryotic microbiology*. 1995;42(2):150-7.
230. Guillermo LV, DaMatta RA. Nitric oxide inhibition after *Toxoplasma gondii* infection of chicken macrophage cell lines. *Poultry science*. 2004;83(5):776-82.
231. Mavin S, Joss AW, Ball J, Ho-Yen DO. Do *Toxoplasma gondii* RH strain tachyzoites evolve during continuous passage? *J Clin Pathol*. 2004;57(6):609-11.
232. Chatterton JM, McDonagh S, Ho-Yen DO. *Toxoplasma* tachyzoites from cell culture are more appropriate in some situations. *J Clin Pathol*. 2010;63(5):438-40.
233. Degirmenci A, Doskaya M, Caner A, Cicek C, Korkmaz M, Guruz Y, et al. *Toxoplasma gondii* RH Ankara: production of evolving tachyzoites using a novel cell culture method. *Experimental parasitology*. 2011;128(1):1-8.
234. Sivrikaya G, Bagriacik EU. [Production of *Toxoplasma gondii* in Vero cell culture]. *Turkiye parazitolojii dergisi / Turkiye Parazitoloji Dernegi = Acta parasitologica Turcica / Turkish Society for Parasitology*. 2011;35(2):61-4.
235. da Costa-Silva TA, da Silva Meira C, Frazzatti-Gallina N, Pereira-Chioccia VL. *Toxoplasma gondii* antigens: recovery analysis of tachyzoites cultivated in Vero cell maintained in serum free medium. *Experimental parasitology*. 2012;130(4):463-9.
236. Klotz C, Aebischer T, Seeber F. Stem cell-derived cell cultures and organoids for protozoan parasite propagation and studying host-parasite interaction. *International journal of medical microbiology : IJMM*. 2012;302(4-5):203-9.
237. Association BD PORC. Rapport du commissaire aux comptes sur les comptes annuels. 2012.
238. Weigel RM, Dubey JP, Siegel AM, Kitron UD, Mannelli A, Mitchell MA, et al. Risk factors for transmission of *Toxoplasma gondii* on swine farms in Illinois. *The Journal of parasitology*. 1995;81(5):736-41.
239. Assadi-Rad AM, New JC, Patton S. Risk factors associated with transmission of *Toxoplasma gondii* to sows kept in different management systems in Tennessee. *Vet Parasitol*. 1995;57(4):289-97.
240. Lehmann TG, D. H.; Dahl, E.; Sreekumar, C.; Launer, F.; Corn, J. L.; Gamble, H. R.; Dubey, J. P. Transmission dynamics of *Toxoplasma gondii* on a pig farm. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases*. 2003;3(2):135-41.
241. Hill DE, Haley C, Wagner B, Gamble HR, Dubey JP. Seroprevalence of and risk factors for *Toxoplasma gondii* in the US swine herd using sera collected during the National Animal Health Monitoring Survey (Swine 2006). *Zoonoses Public Health*. 2010;57(1):53-9.
242. Correa R, Cedeno I, de Escobar C, Fuentes I. Increased urban seroprevalence of *Toxoplasma gondii* infecting swine in Panama. *Vet Parasitol*. 2008;153(1-2):9-11.
243. Association BD PORC. Rapport du commissaire aux comptes sur les comptes annuels. 2013.
244. INSEE. Évolution de la population jusqu'en 2015: Institute nationale de la statistique et des études économiques; 2015 [cited 2015]. Available from: http://www.insee.fr/fr/themes/tableau.asp?reg_id=0&ref_id=NATnon02145%C2.
245. Wang Y, Wang G, Zhang D, Yin H, Wang M. Identification of novel B cell epitopes within *Toxoplasma gondii* GRA1. *Experimental parasitology*. 2013;135(3):606-10.
246. Gomez-Samblas M, Vilchez S, Racero JC, Fuentes MV, Osuna A. Quantification and viability assays of *Toxoplasma gondii* in commercial "Serrano" ham samples using magnetic capture real-time qPCR and bioassay techniques. *Food microbiology*. 2015;46:107-13.
247. Santos SL, de Souza Costa K, Gondim LQ, da Silva MS, Uzeda RS, Abe-Sandes K, et al. Investigation of *Neospora caninum*, *Hammondia* sp., and *Toxoplasma gondii* in tissues from slaughtered beef cattle in Bahia, Brazil. *Parasitol Res*. 2010;106(2):457-61.
248. Villena I, Aubert D, Gomis P, Ferte H, Ingland JC, Denis-Bisiaux H, et al. Evaluation of a strategy for *Toxoplasma gondii* oocyst detection in water. *Appl Environ Microbiol*. 2004;70(7):4035-9.

249. Dubey JP, Weigel RM, Siegel AM, Thulliez P, Kitron UD, Mitchell MA, et al. Sources and reservoirs of *Toxoplasma gondii* infection on 47 swine farms in Illinois. *The Journal of parasitology*. 1995;81(5):723-9.
250. Dumetre A, Darde ML. How to detect *Toxoplasma gondii* oocysts in environmental samples? *FEMS Microbiol Rev*. 2003;27(5):651-61.
251. Hoelzer K, Moreno Switt AI, Wiedmann M. Animal contact as a source of human non-typhoidal salmonellosis. *Vet Res*. 2011;42:34.
252. Davies PR, Morrow WE, Deen J, Gamble HR, Patton S. Seroprevalence of *Toxoplasma gondii* and *Trichinella spiralis* in finishing swine raised in different production systems in North Carolina, USA. *Preventive veterinary medicine*. 1998;36(1):67-76.
253. de Sousa RA, Lemos Jda F, Farias LA, Lopes CD, dos Santos KR. Seroprevalence and risk factors for *Toxoplasma gondii* infection in pigs in southern Piauí. *Rev Bras Parasitol Vet*. 2014;23(1):98-100.
254. Tao Q, Wang Z, Feng H, Fang R, Nie H, Hu M, et al. Seroprevalence and risk factors for *Toxoplasma gondii* infection on pig farms in central China. *The Journal of parasitology*. 2011;97(2):262-4.
255. Villari S, Vesco G, Petersen E, Crispo A, Buffolano W. Risk factors for toxoplasmosis in pigs bred in Sicily, Southern Italy. *Vet Parasitol*. 2009;161(1-2):1-8.
256. Luptakova L, Benova K, Rencko A, Petrovova E. DNA detection of *Toxoplasma gondii* in sheep milk and blood samples in relation to phase of infection. *Vet Parasitol*. 2015;208(3-4):250-3.
257. de Santana Rocha D, de Sousa Moura RL, Maciel BM, Guimaraes LA, O'Dwyer H N, Munhoz AD, et al. Detection of *Toxoplasma gondii* DNA in naturally infected sheep's milk. *Genetics and molecular research : GMR*. 2015;14(3):8658-62.
258. Dubey JP, Jones JL. Comments on "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". *Foodborne pathogens and disease*. 2014;11(6):500-1.
259. Dubey JP, Verma SK, Ferreira LR, Oliveira S, Cassinelli AB, Ying Y, et al. Detection and survival of *Toxoplasma gondii* in milk and cheese from experimentally infected goats. *Journal of food protection*. 2014;77(10):1747-53.
260. Jones JL, Dargelas V, Roberts J, Press C, Remington JS, Montoya JG. Risk factors for *Toxoplasma gondii* infection in the United States. *Clin Infect Dis*. 2009;49(6):878-84.
261. Walsh CP, Hammond SE, Zajac AM, Lindsay DS. Survival of *Toxoplasma gondii* tachyzoites in goat milk: potential source of human toxoplasmosis. *The Journal of eukaryotic microbiology*. 1999;46(5):73S-4S.
262. Innes EA, Bartley PM, Buxton D, Katzer F. Ovine toxoplasmosis. *Parasitology*. 2009;136(14):1887-94.
263. Dubey JP, Sharma SP. Parasitemia and tissue infection in sheep fed *Toxoplasma gondii* oocysts. *The Journal of parasitology*. 1980;66(1):111-4.
264. Jungersen G, Jensen L, Riber U, Heegaard PM, Petersen E, Poulsen JS, et al. Pathogenicity of selected *Toxoplasma gondii* isolates in young pigs. *Int J Parasitol*. 1999;29(8):1307-19.
265. Hill DE, Chirukandoth S, Dubey JP, Lunney JK, Gamble HR. Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine. *Vet Parasitol*. 2006;141(1-2):9-17.
266. Dubey JP, Thulliez P, Romand S, Kwok OC, Shen SK, Gamble HR. Serologic prevalence of *Toxoplasma gondii* in horses slaughtered for food in North America. *Vet Parasitol*. 1999;86(4):235-8.
267. Garcia JL, Navarro IT, Vidotto O, Gennari SM, Machado RZ, da Luz Pereira AB, et al. *Toxoplasma gondii*: comparison of a rhoptry-ELISA with IFAT and MAT for antibody detection in sera of experimentally infected pigs. *Experimental parasitology*. 2006;113(2):100-5.
268. Shaapan RM, El-Nawawi FA, Tawfik MA. Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Vet Parasitol*. 2008;153(3-4):359-62.
269. Dubey JPT, P.; Powell, E. C. *Toxoplasma gondii* in Iowa sows: comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. *The Journal of parasitology*. 1995;81(1):48-53.
270. Khan A, Behnke MS, Dunay IR, White MW, Sibley LD. Phenotypic and gene expression changes among clonal type I strains of *Toxoplasma gondii*. *Eukaryotic cell*. 2009;8(12):1828-36.

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List of Publications

Scientific articles:

1. **Djokic V.**, Blaga R., Aubert D., Durand B., Perret C., Geers R., Ducry T., Vallee I., Djurkovic Djakovic O., Mzabi A., Villena I., Boireau P., 2015. *Toxoplasma gondii* infection in pork produced in France. Parasitology – accepted article
2. Radu Blaga, Dominique Aubert, Catherine Perret, Régine Geers, **Vitomir Djokic**, Isabelle Villena, Emmanuelle Gilot-Fromont, Aurélien Mercier, Pascal Boireau. ANIMAUX RÉSERVOIRS DE PATHOGÈNES POUR L'HOMME – accepted article
3. **Djokic V.**, Blaga R., Rinaldi L., Le Roux D., Ducry T., Maurelli MP, Perret C, Djurkovic Djakovic O, Cringoli G, Boireau P. 2014. Mini-FLOTAC for counting *Toxoplasma gondii* oocysts from cat feces--comparison with cell counting plates. Exp Parasitol. 2014 Dec; 147:67-71. doi: 10.1016/j.exppara.2014.10.009. Epub 2014 Oct 18. PMID: 25448359
4. **Djokic V.**, Klun I, Musella V, Rinaldi L, Cringoli G, Sotiraki S, Djurkovic-Djakovic O. 2014. Spatial epidemiology of *Toxoplasma gondii* infection in goats in Serbia. Geospat Health. 2014 May;8(2):479-88. PMID: 24893025
5. Marković M, Ivović V, Stajner T, **Djokić V.**, Klun I, Bobić B, Nikolić A, Djurković-Djaković O. 2014. Evidence for genetic diversity of *Toxoplasma gondii* in selected intermediate hosts in Serbia. Comp Immunol Microbiol Infect Dis. 2014 May;37(3):173-9. doi: 10.1016/j.cimid.2014.03.001. Epub 2014 Mar 15. PMID: 24698636
6. Djurković-Djaković O, **Djokić V.**, Vujanić M, Zivković T, Bobić B, Nikolić A, Slavić K, Klun I, Ivović V. Kinetics of parasite burdens in blood and tissues during murine toxoplasmosis. Exp Parasitol. 2012 Jul;131(3):372-6. doi: 10.1016/j.exppara.2012.05.006. Epub 2012 May 18. PMID: 22613495

Expert commission participation:

1. Marieke Opsteegh, Miriam Maas, Gereon Schares Joke van der Giessen and consortium: Franz Conraths, Berit Bangoura, Radu Blaga, Pascal Boireau, Isabelle Vallee, **Vitomir Djokic**, Delphine Le Roux, Catherine Perret-Duménil, Tamara Ducry, Henk Wisselink, Jan Cornelissen, Isabelle Villena, Dominique Aubert, Adriana Györke, Vasile Cozma, Viorica Mircean, Anamaria Ioana Paștiu, Anamaria Balea, Zsuzsa Kalmar, Diana Bărburaș, Edoardo Pozio, Furio Spano, Georgina Limon, Milen Georgiev, Damer Blake, Javier Guitian, Javier Dominguez, Frank Katzer, Alison Burrells, Lee Innes, Olgica Djurkovic-Djakovic, Ivana Klun – 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

2. Marieke Opsteegh, Gereon Schares, Radu Blaga, Joke van der Giessen and consortium: Franz Conraths, Pascal Boireau, Isabelle Vallee, **Vitomir Djokic**, Delphine Le Roux, Catherine Perret-Duménil, Tamara Ducry, Berit Bangoura, Henk Wisselink, Jan Cornelissen, Isabelle Villena, Dominique Aubert, Adriana Györke, Vasile Cozma, Viorica Mircean, Anamaria Ioana Paștiu, Anamaria Balea, Zsuzsa Kalmar, Diana Bărburaș, Edoardo Pozio, Furio Spano, Simona Cherchi, Georgina Limon, Milen Georgiev, Damer Blake, Javier Guitian, Javier Dominguez, Frank Katzer, Alison Burrells, Lee Innes, Olgica Djurkovic-Djakovic, Ivana Klun - **EFSA External scientific Report: Experimental studies on *Toxoplasma gondii* in the main livestock species (GP/EFSA/BIOHAZ/2013/01)**
3. Radu Blaga, Catherine Perret, **Vitomir Djokic**, Tamara Ducry, Isabelle Vallée, Pascal Boireau, Dominique Aubert, Régine Geers, Isabelle Villena, Benoît Durand – **DGAL : Contamination par *Toxoplasma gondii* des viandes de boucherie (espèce porcine) Résultats du plan de surveillance 2013**

Oral Presentations on international congresses:

1. **V.Djokic**, G. Limon, C.Perret Duménil, D.Aubert, R.Geers, T.Ducry, B.Durand, P.Boireau, I.Villena, J. Guitian, M. Georgiev, J.Dominguez, J. van der Giessen, R.Blaga,. *Toxoplasma gondii* infection in pigs: correlation between direct and indirect detection methods and on farm risk factors. apiCOWplexa 2015, Edimbourg, UK June 2015
2. **Djokic V.**, Blaga R., Rinaldi L., Le Roux D., Perret C., Ducry T., Maurelli M.P., Durand B., Djurkovic Djakovic O., Cringoli G., Boireau P. *Toxoplasma gondii* oocysts counting by Mini FLOTAC and Kova Slide. 13th INTERNATIONAL CONGRESS OF PARASITOLOGY. Mexico City, Mexico, 10-15/08/2014
3. R.Blaga, C.Perret Duménil, D.Aubert, T.Ducry, R.Geers, M.Thomas, **V.Djokic**, I.Villena, P.Boireau, *Toxoplasma gondii* contamination of imported horse meat. 13th INTERNATIONAL CONGRESS OF PARASITOLOGY. Mexico City, Mexico, 10-15/08/2014
4. Ivovic V., **Djokic V.**, Vujanic M., Djurkovic-Djakovic O. (2012). Current distribution and predicted range expansion of the sand fly vector *Phlebotomus neglectus* (Diptera, Psychodidae) in the Eastern Mediterranean. EUROPEAN MULTICOLLOQUIUM OF PARASITOLOGY – EMOP XI, Cluj Napoca, Romania 25-29.06. 2012

5. **Djokić V.**(2012). Spatial analysis of *Toxoplasma gondii* infection in goats in Serbia. EUROPEAN MULTICOLLOQUIUM OF PARASITOLOGY – EMOP XI, Cluj Napoca, Romania 25-29.06. 2012
6. Nikolić A, Klun I, **Djokić V**, Bobić B, Vujanić M, Živković T, Djurković-Djaković O (2012): Geographical distribution of *Ascaris lumbricoides* and *Trichuris trichiura* in Serbia. 11th European Multicolloquium of Parasitology, Cluj-Napoca, Romania, 25-29.06. 2012
7. **Djokić V**, Nikolić A, Klun I, Bobić B, Ivoić V, Vujanić M, Živković T, Djurković-Djaković O (2010): Application of GIS in mapping geohelminth parasites in Serbia. Geographic Medicine & Global Health – 44th Annual Meeting of the Österreichischen Gesellschaft für Tropenmedizin und Parasitologie – ÖGTP, Graz, Austria, 18-20.11. Abstract book, p.47.
8. **Djokić V**, Klun I, Vujanić M, Ivoić V, Djurković-Djaković O (2011): Mapping of *Toxoplasma gondii* infection in goats in Serbia. FA - Second International Conference of Southeastern and Eastern European Parasitological Society (SEEEP), Zagreb, Croatia, 14-15.06.