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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

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Thèse de doctorat de : Parasitologie

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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:

With my gratitude for their examples, support, tolerance and inspiration throughout my studies

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Résume

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, НСТ-8) и хуманим епителијалним колоректалним ћелијама аденокарцинома (human epithelial colorectal adenocarcinoma cells, СаСо-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 пропацију 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 товљеника, 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 мишјег 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). McNamee 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 узорака паразити нису изоловани. Производња НФФ 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 НФФ 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 week 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 worm-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 rappers 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 unabled, 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-LAT-100%				
MAT	100	87.5	YES	MAT-IHAT-62.5%	24	10 oocysts	1996	Dubey, J. P.
LAT	100	100		MAT-DT-87.5%				Andrews, C. D.
IHAT	100	100						Lind, P.
MAT	100	100		MAT-LAT- 100%				
LAT	80.0	100		MAT-IHAT- 37.5%	24	1 oocysts		Kwok, O. C.
IHAT	100	100		MAT-DT-91.7%				
								Lunney, J. K.
IFAT	87.3	87.0						
WB	93.5	77.2	6	IFAT-WB – 76.8%				
TgSAG1-ELISA	93.1	98.8		IFAT-TgSAG1 – 86.9				
ELISA PrioCHECK	98.9	92.7						
			NO	IFAT-PrioCHECK – 84.8	297	NA	2013	10.1016/j.ijpara.2013.02.003
				WB-TgSAG1 – 83.8				
				WB-PrioCHECK – 81.8				
				TgSAG1-PrioCHECK – 93.9				
ELISA PrioCHECK	100	100	YES		6	5000 oocysts		
Bead-Based Assay	85.5	91.3	NO	BBA – ELISA – 85%	1223	NA	2015	10.1016/j.vetpar.2014.11.020

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
TgSAG2	75.0	96.3	NO	2	LAT-TgSAG2 – 84.7%				
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	YES		NA	51	Gansu Jingtai strain	2013	10.1016/j.exppara.2013.09.019
PG13	100	100							
PG18	100	100							
Bead-Based Assay	86.0 64.0	96.0 95.0		;	BBA-RIVM – kappa 68 BBA-Safepath – kappa 80				
RIVM-ELISA	76.0	93.0			BBA-ID-VET – kappa 93	932	NA	2012	10.1186/1746-6148-8-36
Safepath –ELISA	84.0	99.0	NO		RIVM-Safepath – kappa 75 RIVM-ID-VET – kappa 72 Safepath-ID-VET – kappa 80				
ELISA	sera 100	sera 100							
MAT	100	100	YES		NA	10	100 -1000 oocysts	2012	10.1016/j.vetpar.2012.06.040
ELISA	tissue fluids 71.4	tissue fluids 100	VEG strain						
MAT	100	100							
cELISA	88.1	94.5	NO		MAT-cELISA – 94.5%	861	NA	2011	973070 [pii]

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

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 ;	23.2	99	12	ELISA	Burrells, A., <i>et al</i>	2013	10.1017/s0031182013001169
	...2.1.1.26 red foxes;	6.0	83	1				
	...2.1.1.27 polecats;	31.4	70	9				
	...2.1.1.27 polecats;	29.2	65	6				
	...2.1.1.27 polecats;	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				
		red deer; red fox	0	7	0				
			NA	304	57				
	Red fox		38.0	221	NA	DAT	Jakubek, E.B., <i>et al</i>	2001	
Sweeden	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	116	14	ELISA	Jokelainen, P., <i>et al</i>	2011	10.7589/0090-3558-47.1.154
			4.0	99	4				
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	353	48	Immunoblot	Herrmann, D. C., <i>et al</i>	2012	10.1016/j.vetpar.2011.10.030
			0		0				
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	
	...2.1.1.35	Anas platyrhynchos							
	...2.1.1.36	Anas clypeata	11.8 18.2 7.3	17 11 41	3	NA	Mancianti, F., <i>et al</i>	2013	10.1645/12-34.1
Italy	...2.1.1.37	Anas crecca	25.0	8					
	...2.1.1.38	Gallinago gallinago							
	Roe deer Chamois	13.0 5.0	207 108	NA	LAT	Gaffuri A., <i>et al</i>	200-	10.7589/0090-3558-42.3.685	
	...2.1.1.39	Striped dolphin	11.1 50.0 57.1	36 4 7	NA	MAT	Cabezon, O., <i>et al</i>	2004	10.1645/ge-257r
Spain	...2.1.1.40	Commo	0	9					

	n dolphin							
...2.1.1.41	Bottlenose dolphin							
	Rissos's dilphin							
...2.1.1.42	red deer;							
...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.2005.11.013
...2.1.1.46	chamois	10 14.8	10 27					
...2.1.1.47	Barbary sheep							
...2.1.1.48	mouflon							
Wild boar		38.4	507	NA	MAT	Gauss, C.B.L., <i>et al</i>	2005	10.1016/j.vetpar.2005.04.023
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.2007.06.038
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	Cabezon, 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	Buteo	79	14	0				
	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	NA	NA	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			1501	48.6	NA					
	Goats	69	98.6	541	30.7	NA	ELISA	Tzanidakis, N., <i>et al</i>	2012		10.1016/j.vetpar.2012.07.020
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					
	Sheep	NA	NA	250	61.6	NA	ELISA	Berger-Schoch, A. E., <i>et al</i>	2011		10.1111/j.1863-2378.2011.01395.x
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					
	Sheep	NA	NA	511	84.5	NA	MAT	Klun, I., <i>et al</i>	2006		10.1016/j.vetpar.2005.08.010
	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			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
	Cattle	NA	NA	865	14.6	NA	cELISA	Sroka, J., <i>et al</i>	2011	973070 [pii]
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	12	type II				
	...2.1.1.54	polecat s;	83	1	PCR, RFLP				types I and III, type II
			70	9					
			65	6					
...2.1.1.55	minks; badgers; stoats	64	2	type II					
	rabbit	9	0						
		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;	73	1	PCR, RFLP	De Craeye, S., <i>et al</i>	2011	10.1016/j.vetpar.2010.12.016	
	...2.1.1.61	bred fallow deer;	4	0					NA NA NA 25 type II, 1 type III
			7	0					
	red deer; red fox	304	57						
Sweden	...2.1.1.62	Brown rat	49	0	PCR	NA	Backhans, A., <i>et al</i>	2012	
			86	0					
			12	0					

	...2.1.1.63	House mouse								
	...2.1.1.64	Yellow necked mouse								
Finland	brown hares;		116	14	PCR,	14 type II,		Jokelainen, P., <i>et al</i>	2011	10.7589/0090-3558-47.1.154
	mountain hares		99	4	microsatellites	4 type II				
Germany	red fox;		353	48	PCR, RFLP	13 type II, 4 type II clonal		Herrmann, D. C., <i>et al</i>	2012	10.1016/j.vetpar.2011.10.030
	rodents			0						
	beavers;		6	2	PCR, RFLP	2 type II;		Herrmann, D. C., <i>et al</i>	2013	10.1016/j.vetpar.2012.08.026
	wildcats		12	4		4 type II				
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				Hurkova, L. and Modry D.	2006	10.1016/j.vetpar.2006.01.005
	martens;		61	3						
	badgers;		19	0	PCR	NA				
	raccon dogs;		3	0						
	polecats;		2	0						
ferrets;		2	0							
	otter		1	0						
Austria	<i>C. glareolus</i> ;		40	0				Schmidt, S., <i>et al</i>	2014	10.1089/vbz.2013.1504
	<i>Apodemus flavicollis</i> ;		29	0	PCR	NA				
	<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	3				Ferroglia, E., <i>et al</i>	2014	10.1186/1756-3305-7-196
			105	17	PCR	NA				
			94	19						
			22	0						

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

	<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
	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
France	<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			Aubert, D. <i>et al</i>	2010	10.1016/j.vetpar.2010.03.033
	<i>Capreolus capreolus</i>	60	12		12 type II			
	<i>Ovis gmelini</i>	31	1	Bioassay,	1 type II			
	<i>Cervus elapus</i>	24	1	RFLP,	1 type II			
	<i>Dama dama</i>	4	0	microsatelites				
	<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 foetuses	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

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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**

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41 1.1. SUMMARY

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

55
56 Key words:

57
58 *Toxoplasma gondii*, pork, intensive farms, outdoor farms, seroprevalence, parasite isolation

59 60 KEY FINDINGS

- 61 • This study reports on an innovative survey of *Toxoplasma gondii* infection in pigs from
62 France, born and raised on intensive and outdoor farms.
- 63 • Seroprevalence, strain isolation and risk-factor analysis were assessed based on production
64 systems, age categories and regions of slaughtering.
- 65 • The adjusted seroprevalence in pigs from intensive farms was 3.0%, highest in sows (13.4%),
66 2.9% in fattening pigs, and 2.6% in piglets. Outdoor farm seroprevalence in fattening pigs was 6.3%.
- 67 • Forty-one strains were isolated (25 from intensive and 16 from outdoor farms) and all strains
68 were genotyped as type II.
- 69 • Risk-factor analysis showed an above three-fold (3.62) increased the risk of infection for pigs
70 raised on outdoor farms ($P=0.004$), and that sows' risk of infection is nearly five times (4.63) higher
71 than that of fattening animals ($P<0.001$).

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

76

77 **1.2. INTRODUCTION**

78 With the widest range of mammalian hosts, including humans, *Toxoplasma gondii* is the most
79 unrestricted parasite on Earth, infecting one third of the global human population (Torrey & Yolken,
80 2013). The organism has a complex life cycle that can involve all mammals and birds as intermediate
81 hosts (by clonal division) and members of the Felidae family as definitive hosts (by reproduction). In
82 immunocompetent individuals, *T. gondii* generally causes mild and self-limited infection. However, in
83 categories of the population with a compromised immune system, such as foetuses and
84 immunosuppressed individuals, the parasite may cause life-long infection of the nervous system and
85 eyes, or life-threatening disease.

86 Human infection occurs through the ingestion of tissue cysts, present in contaminated meat, or oocysts
87 (excreted by felids), via contaminated water, fruits and vegetables. A recent study has predicted the
88 prevalence of *T. gondii* at 27% among pregnant French women until 2020 (Nogareda et al., 2014).
89 Along with sheep, pigs are the species mostly associated with human infection (Esteban-Redondo et
90 al., 1999). Consumption of infected undercooked meat or meat products is considered a major risk
91 factor, especially in Europe, which has accounted for 30–63% of infections (Bobic et al., 1998; Cook
92 et al., 2000; Tenter et al., 2000). Pigs have traditionally been an important source of meat in many
93 developing countries, and are the only species shown to regularly harbour the parasite (Hill & Dubey,
94 2013). Bradyzoites in tissue cysts have been experimentally shown to survive in meat stored at
95 temperatures below 0°C for 7 days (Hill et al., 2006), and at +58°C for only 9.5 minutes (Dubey et al.,
96 1990), as well as at pressures below 300–400 Mega Pascal (Lindsay et al., 2006). Chemical processing
97 of pork in 2% sodium chloride and 1.4% potassium or sodium lactate inactivates parasites within 8
98 hours (Hill et al., 2006) by reducing the quantity of free water (water activity index – aw). Thus,
99 inappropriate and insufficient use of these methods allows the parasite to stay infective in meat
100 products, or even after preparation of pork for direct consumption.

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

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

120

121 1.3. MATERIALS AND METHODS

122

123 1.3.1. Sampling strategy

124 In the Netherlands in 2007 different pig farming types were studied independently and the
125 prevalence of 1% on intensive and 5% on outdoor farms was showed (van der Giessen et al. 2007).
126 Based on the breeding practices reported in the Netherlands and France, the estimation was that *T.*
127 *gondii* prevalence in France could correspond to that of the Netherlands. According to the annual pork
128 production report (Association BD PORC, 2012) the number of samples originating from intensive
129 farms was set at 1,500, with 300 samples coming from outdoor farms. A stratified sampling strategy
130 was devised. Based on the national report of pork production in France for 2012 (Association BD
131 PORC, 2012), eight regions were chosen (Fig 1), representing 92% of the annual pork production in
132 intensive (6 regions) and in outdoor farms (7 regions). In order to obtain the same precision of
133 prevalence estimates on a national level, the same number of samples was collected from each region;
134 215 from intensive and 43 from outdoor farms, respectively. Next, two levels of stratification were
135 formed: two production types and three age categories. The three age categories were defined as
136 piglets (up to 25kg or 2 months of age), fattening pigs (100-110kg or 8 months of age) and older,
137 breeding pigs-sows. From intensive farms, 115 fattening pigs, 50 piglets and 50 sow samples were
138 collected per region. From outdoor farms, 23 fattening pigs, 10 piglets and 10 sow samples were
139 obtained from each region.

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

143

144 **1.3.2. Sampling protocol**

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

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

158

159 **1.3.3. Serology test**

160 Detection of *T. gondii* antibodies was performed on cardiac fluids by the modified agglutination
161 test (MAT), as described by Villena et al (2012). This is a species-independent serological test,
162 considered to be the gold standard for the detection of *T. gondii* antibodies in animals and meat (Klun
163 et al., 2006). The antigen was provided by the National Reference Centre for toxoplasmosis in Reims,
164 France. The starting dilution was 1:6, in accordance with a previous study in sheep (Halos et al.,
165 2010). Six further two-fold dilutions were made, up to 1:200. All samples reactive at $\geq 1:6$ were
166 considered positive.

167

168 **1.3.4. Mouse bioassay and qPCR**

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

174 peritoneal inoculation into two Swiss-Webster mice (CD-1) in bioassay (1 ml per mouse. Ethics
175 committee licence no: 21/01/13-2). Mice were bled after 6 weeks from the retro orbital sinus and
176 tested by MAT for *T. gondii* antibodies. All seropositive mice were euthanized and brains were
177 collected aseptically. Samples of whole brain homogenates were microscopically examined for *T.*
178 *gondii* cysts. Of the brain homogenates, 300 µl were used for DNA extraction. All DNA extractions
179 were done using QIAmp mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's
180 instructions. Quantitative Polymerase Chain Reaction (qPCR), targeting the 529 bp, repetitive element
181 (gene bank accession number AF146527) was performed as previously described by Vujanic et al.,
182 (2011).

183

184 **1.3.5. Genotyping of *T. gondii* isolates**

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

188

189 **1.3.6. Definition of cases**

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

192

193 **1.3.7. Statistical analysis**

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

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

212

213 **1.4. RESULTS**

214

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

218

219 **1.4.1. Collected samples**

220 The study was conducted on a total of 1,549 pigs (Table 7), from 752 farms. Of the 1,549, 1,342
221 (86.7%) were intensively bred animals, and 207 (13.3%) were outdoor pigs, which is proportional to
222 the number of animals slaughtered from each rearing system in France. According to age, samples
223 were collected from 133 (8.6%) piglets, 1,158 (74.7%) fattening (market weight) pigs, and 258
224 (16.7%) sows (Table 7), which matches with the number of pigs slaughtered in each of these age
225 categories in the country. The number of samples from individual farms varied between one and 25
226 animals, and depended on the production type, age category and size of the originating farm. Since the
227 study was based on the numbers of animals slaughtered per region, the seroprevalence results were
228 further analysed according to the region of slaughtering.

229

230 **1.4.2. Seroprevalence analysis**

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

233

234 **1.4.3. Seroprevalence in pigs from intensive farms**

235 The adjusted seroprevalence of *T. gondii* infection in pigs raised in intensive farm systems was
236 2.7% (95% CI: 0.0-7.0%). The highest seroprevalence was in the Bretagne region (6.3%, 95% CI: 3.3-
237 9.3%) and the lowest in Basse – Normandie (1.6%, 95% CI: 1.0-5.0%) (Fig 2). The highest adjusted
238 seroprevalence was found in sows (13.4%, 95% CI: 6.8-19.9%), followed by fattening pigs (2.9%,
239 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).

240

241 **1.4.4. Seroprevalence in pigs from outdoor farms**

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

247

248 **1.4.5. Live parasite isolation**

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

254 Bio-assays were performed on 11 piglet samples, but no strain was identified. Various *T. gondii*
255 isolates were found in 27 out of 98 fattening pigs (27.55%) and 14 out of 51 sows (27.45%). From the
256 27 strains found in fattening pigs, 12 originated from intensive and 15 from outdoor farms. The
257 majority of strains isolated from sows, 13 of them, originated from intensively bred animals (but 49
258 samples were tested), and only one came from an outdoor animal (only 2 samples were tested) (Table
259 7).

260 When adjusted according to the total number of animals in each age category, the strain
261 prevalence in intensive fattening pigs was 0.9% (95% CI: 0.0-1.9%) and 8.8% in sows (95% CI: 3.2-
262 14.3%). In outdoor fattening animals the overall strain presence was 10.2% (95% CI: 0.0-22.1%).

263

264 **1.4.6. Concordance between serology and parasite isolation**

265 Out of 160 bio-assays, 41 strains were found. Substantial concordance between serology and the
266 presence of infective parasites was calculated (Kohen's kappa 0.66, 95%CI: 0.52-0.80). All digested
267 hearts were tested in bio-assay and examined for *T. gondii* specific DNA by qPCR. Thirty-four out of
268 160 found positive (between 20th and 35th cycle), indicating moderate concordance between serology
269 and PCR (Kohen's kappa 0.58, 95%CI: 0.43-0.72). Moderate accord was also obtained between two
270 direct detection methods: bioassay and PCR (Kohens's kappa 0.58, 95%CI: 0.43-0.72).

271 **1.4.7. Risk-factor analysis**

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

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

282

283 **1.5. DISCUSSION**

284

285 There is numerous data on the seroprevalence of *T. gondii* infection in pigs throughout the world,
286 with values ranging from 0.9% in sows from Austria (Edelhofer, 1994) to 0% in pork from the
287 Netherlands (Kijlstra et al., 2004), to as high as 45.3% in organic farms from Veracruz, Mexico
288 (Alvarado-Esquivel et al., 2014) and 37.8% in Argentina (Venturini et al., 2004) (Table 9). The live
289 parasites are isolated from pigs in all parts of the world with types II and III predominant in Europe
290 and North America (Mondragon et al., 1998; Klun et al., 2011; Dubey et al., 2012; Turcekova et al.,
291 2013), and atypical strains more characteristic for South America and Asia (Zakimi et al., 2006;
292 Belfort-Neto et al., 2007; Zhou et al., 2010b; Bezerra et al., 2012).

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

299 This is the first nation-wide study in France that uses pork production from both systems (indoor
300 and outdoor) and consumption data to construct the sampling strategy. The results of this cross-
301 sectional survey of *T. gondii* infection in pigs from France show adjusted prevalence values of 3.0% at
302 intensive and 6.3% at outdoor farms. Studies in Spain and Portugal, using the same test (MAT),
303 showed a prevalence of 16.6% and 9.8% respectively (Garcia-Bocanegra et al., 2010; Lopes et al.,
304 2013) while in Switzerland, using an Enzyme-linked immunosorbent assay (ELISA), Berger-Schoch

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

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

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

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

340 seronegative animal. Similar results were reported from Portugal, Ireland and Slovakia (Halova et al.,
341 2013; Turcekova et al., 2013; Esteves et al., 2014) and even the USA (Lehmann et al., 2003; Dubey et
342 al., 2012). At the opposite, 13 positive qPCRs were obtained from seronegative animals, but without
343 strain isolation. This can be interpreted as a lack of sensitivity of available detection methods.
344 Considering the fast fattening period, the time between pig infection and the development of an
345 immunological reaction could be quite limited and undetectable using existing methods. Thus, new
346 diagnostic tools are needed in order to detect early infections in pigs and allow for more accurate risk-
347 assessment studies. RFLP genotyping showed that type II strains are 100% prevalent among pigs in
348 France, which corresponds with the majority of strains found in sheep (Halos et al., 2010) and bovines
349 slaughtered in France (unpublished data). Type II was also the most prevalent in other European
350 countries: pigs from Portugal and Slovakia (de Sousa et al., 2006; Turcekova et al., 2013), sheep from
351 Switzerland and Serbia (Frey et al., 2012; Markovic et al., 2014) but also cats from Germany (Schares
352 et al., 2008).

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

362

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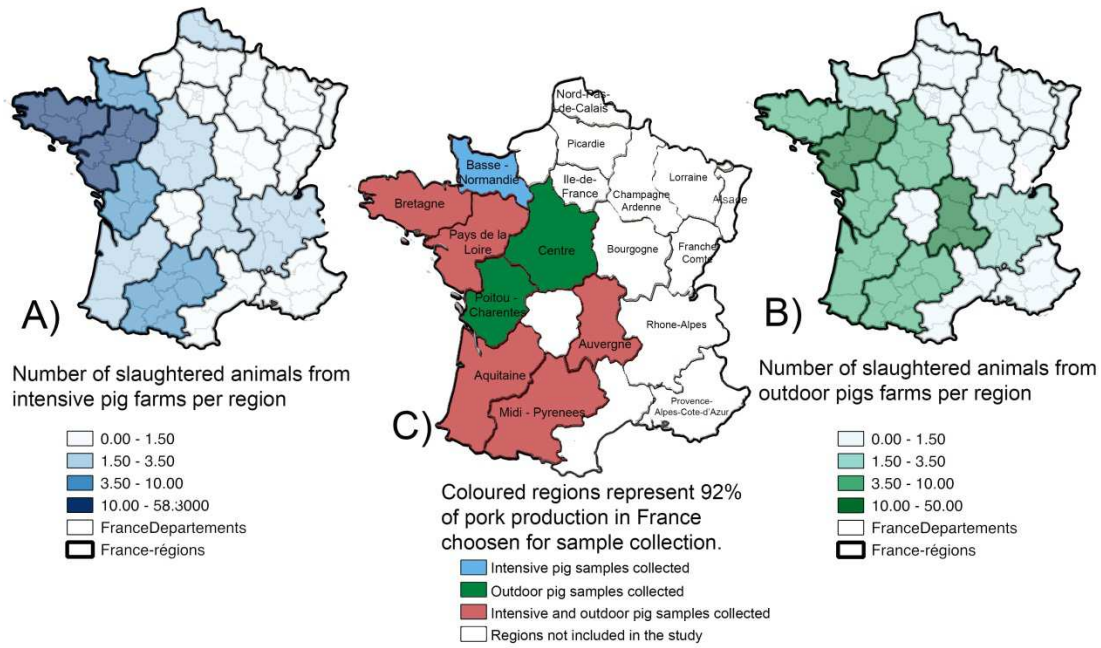
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367

368

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

371 Shades of blue (intensive pig farms) and green (outdoor pig farms) represent scale of regional pork
 372 production in France. Eight regions, coloured on map C in blue, green and red, were selected for study
 373 rendering 92% of all animals slaughtered in 2012.
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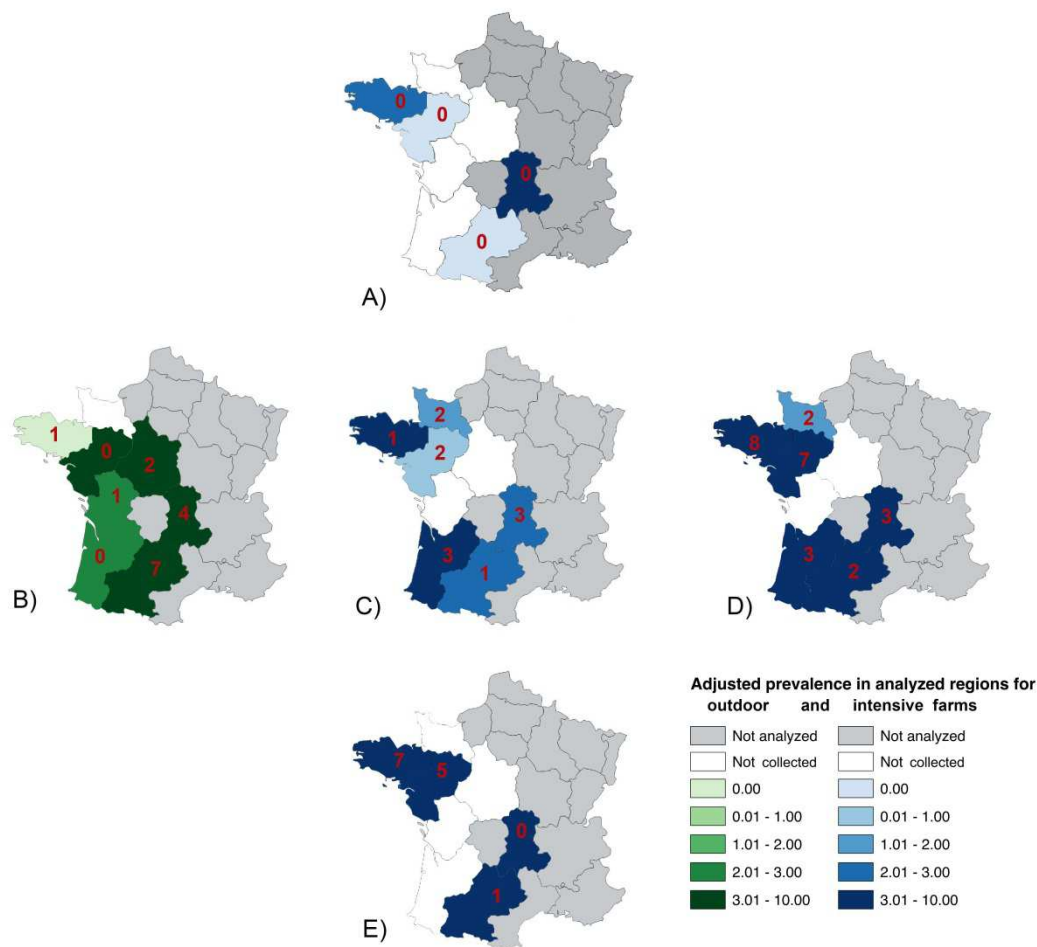
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385 **Figure 2** *Toxoplasma gondii* adjusted seroprevalence and strain isolations according to the
 386 production and age categories in analysed regions

387 Adjusted Seroprevalence (calculated in percentage) is presented according to: 1. Production
 388 categories: outdoor pig farming– B (green scale) and intensive pig farming – A, C, D, E (blue scale);
 389 2. Age categories: A – piglets, B and C - fattening pigs, E - sows. Numbers of strains isolated from
 390 each category per region are written in red. For intensively bred pigs the sum of all age classes is
 391 calculated on map D. Comparison was made only between B and C where there is a noticeably higher
 392 prevalence in pigs from outdoor than indoor farms. The highest number of strains originates from
 393 fattening pigs bred in outdoor farms.
 394



395

396

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	n	Prevalence (%)	Univariate analysis				Multiple model ⁴¹⁰		
			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
<u>Europe</u>								
(Edelhofer, 1994)	Austria	NA	NA	IFAT	NA	0.9	NA	4697
(van der Giessen et al., 2007)	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	
			Breeding		13.2		53	
(Garcia-Bocanegra et al., 2010)	Spain	NA	Fattening Sows	MAT	9.7	16.6	1570	2970
					24.2		1400	
(Berger-Schoch et al., 2011; Frey et al., 2012)	Switzerland	indoor	Finishing Adults	ELISA	14.0	23	50	270
		outdoor	NA		36.0		120	
					13.0		100	
(Bartova and Sedlak, 2011)	Czech Republic	NA	Fattening	ELISA	NA	36	NA	551
(Pastiu et al., 2013)	Romania	Indoor	Fattening Sows	IFAT	0	30.5	660	3595
		outdoor	NA		12.4		371	
					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	
			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		14.9		114	
			Fattening	ELISA	25.2	27.0	671	1022
			Breeding		38		237	
(Wu et al., 2012)	Tibet	Outdoor	Piglets		21.1		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.

1 **2. Section 2:**

2 Paper submitted to Parasites & Vectors journal

3 **Factors associated with *Toxoplasma gondii* infection in confined**
4 **farrow-to-finish pig herds in Western France: an exploratory study in**
5 **60 herds**

6
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21

22 **2.1. Summary**

23 **Background**

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

29 **Methods**

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

35 **Results**

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

41

42 **Conclusions:**

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

48

49 **Key words:**

50 *Toxoplasma gondii*, intensive pig farm, risk factors

51

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.

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

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

96 **2.3. Materials and methods**

97

98 **2.3.1. Database and serum bank**

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

108

109 **2.3.2. Herd selection and risk factors**

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

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

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

132

133 **2.3.3. Serology testing**

134 Detection of *T. gondii* IgG antibodies was performed on sera by the modified agglutination test
135 (MAT), as described by Halos, Thebault (47), since this is a species independent serological test.
136 MAT is considered as gold standard for the detection of *T. gondii* antibodies in animals and meat (54,
137 62, 78, 112). The National Reference Centre for toxoplasmosis in Reims, France provided the antigen.
138 Four two-fold dilutions were made starting from 1/6 to 1/50. All results were observed qualitatively
139 (negative/positive) regardless the dilutions.

140

141 **2.3.4. Statistical analysis**

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

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

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

155

156 **2.4. Results**

157

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

161

162 **2.4.1. Animal, farm and within-farm levels of *T. gondii* frequency of 163 seropositivity**

164 All herds had at least one seropositive pig, thus making the frequency of seropositive farms at
165 100% (95% CI: 94%-100%). When only one age category was observed, farm prevalence in suckling
166 piglets (<1month) was 50% (95% CI: 33%-57%), as well as in weaned animals (1-2 months old -
167 48%, 95%CI: 27-69%), and 2-3 months old fattening pigs (48%, 95% CI: 35%-62%). However, farm
168 prevalence was higher in pigs older than 3 months: 82% (95% CI: 70%-90%).

169 The overall animal-level seroprevalence was 7.0% (95% confidence interval [CI]: 6.2%-
170 7.9%), and varied according to the age class: 5.3% in <1 month old suckling piglets (95% CI: 3.6%-
171 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
172 old (95% CI: 7.6%-11.7%), and 6.0% in animals older than 3 months (95% CI: 4.9%-7.1%) (Table
173 10).

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

179 **2.4.2. Risk factor analysis**

180 The variable selection procedure led to the inclusion of 5 risk factors in the multivariate mixed
181 model: the age class, the herd size (number of sows on a logarithmic scale), the use of specialised
182 clothes inside production units, the presence of a Danish entry, and the presence of dairy cattle (table
183 10).

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

186 p=0.04,) and for 2-3 months old fattening pigs (OR=2.0, p=0.002) when compared to suckling animals
187 (<1 month of age). However, seroprevalence in >3 months pigs was not significantly different from
188 the one observed in <1 month piglets (p=0.5) (Table 11). Increasing herd size had a significant
189 protective effect on seroprevalence, with an OR of 0.87 (p=0.04) for an increase from the 25%
190 percentile (139 sows/farm) of the distribution to the 75% percentile (251 sows/farm). Dairy cattle
191 presence on the same farm had no influence on *T. gondii* infection in pigs (p>0.05). The presence of
192 Danish entry had a protective effect with an OR of 0.58 (p=0.04) (Table 11).

193

194 2.5. Discussion

195

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

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

221 respiratory diseases and *T. gondii* circulation (other than hygienic conditions which were explicitly
222 considered in the present study). Indeed, the animal-level seroprevalence of 7.0% reported here
223 corresponds to those reported in Serbia, Portugal and Austria, in pigs from same type of confinement
224 (45, 120, 122).

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

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

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

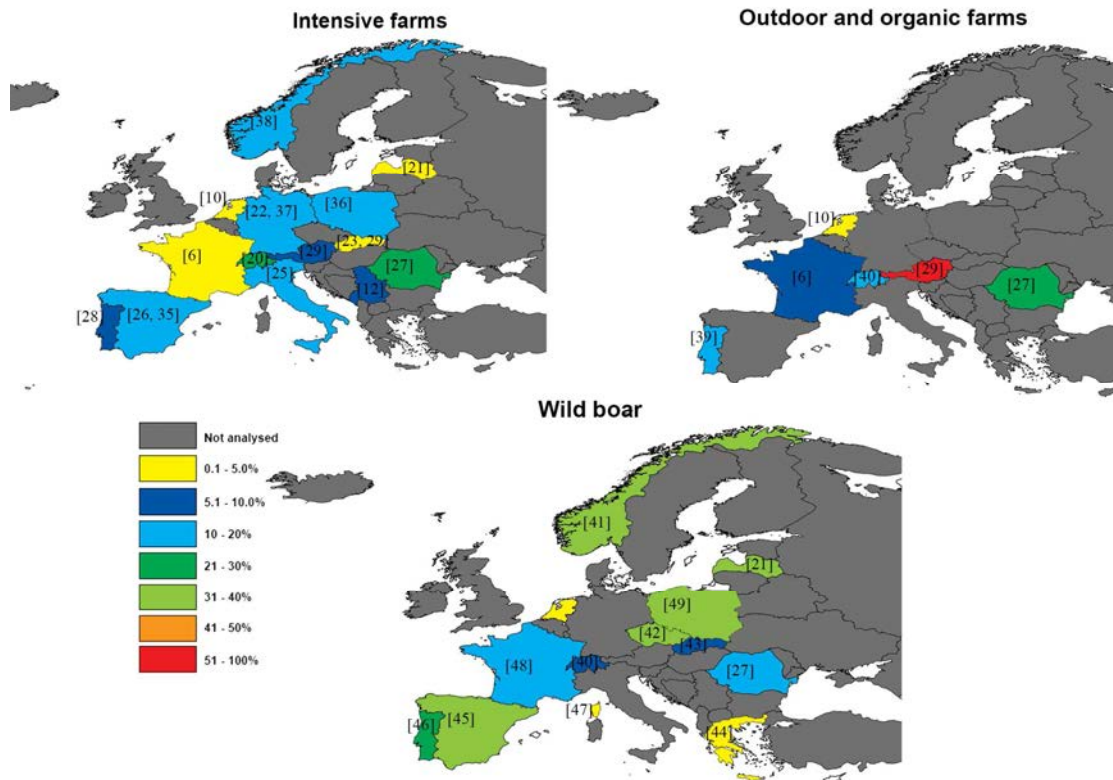
254 **2.6. Conclusion**

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

267

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

269 The results of *T. gondii* investigation in wild boar and pigs from organic/ outdoor farms show small
270 differences in seroprevalence. The seroprevalence ranges from 0.5% in wild boar from island of
271 Corsica to 63% on three organic farms in Austria. These results show those wild boars are important
272 reservoirs for the parasite and that both organic and outdoor farming system do little to prevent
273 infection in domestic pigs. Therefore, meat from these animals represents one of the major sources for
274 human health as well. Used literature: intensive farming: (43, 45, 78, 79, 86-90, 108, 117-119, 121,
275 127, 128); outdoor/organic farming: (45, 87, 90, 108, 129, 130); wild boar: (119, 131-139) [25, 41-49]



276

277

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

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

283

	Risk factor	N	Animals - positive/total (%)	²⁸⁴ p- value
	Dairy production			<0.001
	No	54	207/3236 (6.4%)	286
	Yes	6	44/359 (12.3%)	
	Number of sows per farm¹			<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%)	
Farm level factors	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%)	
	Age in days			0.002
Individu al level factors	≤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

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

300

301

302

303

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305

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

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

312

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

313

314

315 **3. Section 3**

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

318

319 **3.1. Summary**

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

345

3.2. Introduction

346 *Apicomplexa* parasite *Toxoplasma gondii* is the World wide spread food-borne pathogen. Humans
347 and animals, postnatally acquire infection mainly by ingestion of environmentally resistant forms -
348 oocysts, or tissue forms – cysts. Although generally mild in cases of compromised immune system,
349 infection can have serious consequences in i) developing foetus upon maternal infection in pregnancy;
350 and ii) in immunosuppressed individuals; causing the disease – *Toxoplasmosis* (4).
351

352 Pork is considered to be an important source of *T. gondii* infection (95, 140). In the USA pork and
353 *T. gondii* are ranked second among top 10 pathogen-food combinations in terms of annual disease
354 burden (141). *T. gondii* seroprevalence in pigs varies throughout the world ranging from 0% in Brazil
355 – Minas Gerais region, Malaysia and China (142-144), to 53.4% in China– Zheijang province and 60-
356 65.8% in Brazil – Rio de Janeiro and Belem regions (145). In Europe, the prevalence ranges between
357 0.3% in intensively bred pigs from Netherlands to 63.5% in animals bred on organic farms in Austria
358 (45, 87). In South and Central America, strain presence was detected in seropositive pigs between 14.3
359 and 55.3% of the cases, although the seroprevalence in these studies varied from 17.1%-36% (146-
360 149). In Europe out of 14% seropositive pigs in Switzerland PCR detected 0 positives, and in the same
361 time DNA was found in 21 out of 21 ELISA positive animals in Slovakia (89, 150). In four European
362 studies that used bioassay, 26.7% of seropositive intensively bred pigs from Serbia harboured
363 parasites, while in 88.9% of outdoor animals in France *T. gondii* was isolated (108, 120). In the same
364 time live parasites or parasitic DNA were found in raw and cured pork across the World (38, 39, 140,
365 151-155). These data suggest that there is a positive relationship between detection of antibodies in
366 sera and presence of viable tissue cysts of *T. gondii* in the pork. Nevertheless, the overall parasitic
367 burden in pork and percentage this meat makes on the world markets is unknown (10). Although
368 ELISA and IFAT tests showed higher sensitivities and specificities for detection of specific anti - *T.*
369 *gondii* antibodies in pig sera (11, 60, 112), MAT on tissue fluids was in higher concordance with
370 isolation of infectious parasites (62, 156). In the same time, when parasitic DNA is found in the meat,
371 real risk for human infection with *T. gondii* has not been assessed. Both mouse and cat bioassays
372 require specialized animal facilities and strained stuff, are time consuming and expensive to perform.
373 In order to facilitate quantitative screening of large numbers of pork samples Opsteegh, Langelaar
374 (157) developed technique based on magnetic capture of parasitic DNA and quantitative PCR (MC-
375 PCR). MC-PCR performed on diaphragm samples from experimentally infected pigs showed high
376 concordance with mouse bioassay, but has not been tested on the samples of naturally infected pigs.
377 Therefore, the aim of this study was to: i) compare detection of specific IgG anti- *T. gondii* antibodies
378 by modified agglutination test (MAT) in pig sera and cardiac juices; ii) assess the correlation between
379 detection of *T. gondii* antibodies by MAT in two matrices (serum and cardiac juice) and either
380 infective parasites or parasitic DNA detection by mouse bioassay qPCR on tissue digests and magnetic
381 capture PCR (MC-PCR) on diaphragm tissue; iii) examine concordance levels between mouse

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

385

386 **3.3. Materials and Methods**

387

388 **3.3.1. Pig samples**

389 In 2013 nationwide study of *T. gondii* prevalence in pork from French intensive and outdoor
390 farms was conducted (108). Briefly, 1549 pig heart samples (200g), and corresponding 1066 blood
391 (50ml), as well as 1450 diaphragms (100g) were obtained from 26 slaughterhouses throughout 8
392 French regions. Out of this sample base, direct isolation of parasites or parasitic DNA was tried from
393 hearts and diaphragms of 160 pigs.

394

395 **3.3.2. Indirect detection test - MAT**

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

406

407 **3.3.3. Direct detection of infectious parasites (mouse bioassay) or parasitic** 408 **DNA (qPCR and MC-PCR)**

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

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

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

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

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

425

426 *3.3.4. Statistical analysis*

427 To calculate concordance (qualitative agreement - positive/negative) beyond chance between the
428 concentration of IgG antibodies determined by MAT in sera and cardiac juice Cohen's kappa test with
429 95% confidence intervals was used. The same analysis was performed to analyse accordance between
430 MAT and direct detection of parasites in pig tissues (mouse bioassay, qPCR and MC-PCR). For this
431 comparison three direct detection methods were considered separately and as a combined variable of
432 overall direct detection positive animals. Six different concordance categories were determined as
433 described by Landis and Koch (158): i) < 0.00: no concordance; ii) 0.00 – 0.20: poor; iii) 0.21- 0.40:
434 fair; iv) 0.41 – 0.60: moderate; v) 0.61 – 0.80: substantial; vi) 0.81-1.00: almost perfect. MAT is a
435 qualitative test for diagnostic of IgG antibody concentrations. Therefore, to determine the most
436 sensitive matrix for diagnostic of parasite infection in pigs, correlation (agreement among all 7
437 dilutions, each observed separately) between MAT results on sera and cardiac meat juice Pearson's
438 product moment correlation test was used with the same agreement interpretation categories as for
439 Cohen's Kappa. Sensitivity, specificity, positive and negative prediction values and likelihood ratios
440 of MAT on both sera and cardiac juices was calculated on first 3 dilutions (1:6, 1:12 and 1:25) with
441 each direct detection method as reference in separate analysis. Sensitivity and specificity of MAT on
442 two pork fluids showed precision and predictive values in comparison to mouse bioassay, qPCR and
443 MC-PCR. In order to determine the best cut off for MAT receiver operating characteristics analysis
444 was performed on all three matrices separately with results of bioassay as reference (pROC package).

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

449

450 3.4. Results

451

452 3.4.1. MAT concordance and correlation between sera and cardiac juice

453 The results of *T. gondii* - specific IgG detection by MAT on two different matrices are
454 summarized in Table 12. The concordance and correlation analysis has been performed pairwise for
455 118 samples. Fair kappa values were observed between sera and cardiac juice (0.35; 95%CI: 0.18 –
456 0.52) (Table 13). In the same time, by analysing qualitative values of MAT results on two matrices,
457 almost perfect correlation was observed between sera and cardiac juice (0.8; 95%CI: 0.73 – 0.86),
458 with more discordant results on lower dilutions (1:6, 1:12, 1:25 and 1:50) with 56 discordant out of 85
459 samples tested. Higher correlation is observed between MAT on cardiac juice and MAT +1 dilution
460 (ig 1:6 and 1:12) on sera.

461

462 3.4.2. Direct detection of *T. gondii* in pigs

463 The presence of infectious *T. gondii* was demonstrated in 41 out of 148 pig hearts (27.7%), by
464 mouse bioassay. Out of the 159 samples tested by qPCR, 69 (43.4%) had a positive amplification of *T.*
465 *gondii* specific 529bp repetitive element. MC-PCR targeting the same, 529bp fragment of *T. gondii*,
466 found parasite's DNA in 27 (16.9%) pig diaphragms. Four pigs were only bioassay positive, while
467 parasitic DNA was demonstrated in 37 animals, with negative parasite isolation results. Five pigs were
468 proven positive only by MC-PCR, 29 only by qPCR (Figure 4). Presence of *T. gondii* was
469 demonstrated with two diagnostic techniques in total of 26 pigs, while 14 animals were diagnosed
470 with infectious parasites and parasitic DNA by all three direct detection techniques (Figure 4). In total
471 99 samples had concordant result in mouse bioassay and qPCR with recovery rate of 0.74 from
472 positive and 0.16 from qPCR negatives. This resulted in moderate kappa value of 0.5 (95%CI: 0.35-
473 0.66). Kappa value between MCPCR and bioassay had fair value of 0.36 (95%CI: 0.16-0.56) with
474 almost exact recovery rate from both MCPCR positives (0.82) and negatives (0.82).

475

476 3.4.3. Sensitivity and specificity of MAT on sera and cardiac juices

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

482 Results of MAT analyses on sera and cardiac juice were compared to direct detection methods.
483 Since for MAT two fold dilutions, starting with 1/6 (1/6, 1/12, 1/25, 1/50, 1/100, 1/200) was made,
484 sensitivities and specificities of MAT on both matrices were calculated for dilutions of 1/6, 1/12 and
485 1/25 separately. Results of mouse bioassay, qPCR and MCPCR were used as references for sensitivity
486 and specificity calculations (Table 14). The lowest sensitivity was recorded for MAT on sera dilution
487 1/6 when MC-PCR was a reference (Se=0.286), while highest was calculated for MAT on cardiac
488 juice dilution 1/25 when mouse bioassay was set as referent (Se=0.935) (Table 14). MAT on cardiac
489 juice dilution 1/25 showed lowest specificity for qPCR results (Sp=0.517), while the highest result
490 was calculated for sera on 1/6 and 1/12 dilutions (Sp=0.969). Overall, highest values of sensitivity and
491 specificity were shown by MAT on cardiac juices when first two dilutions were used (1/6 and 1/12)
492 (Se=0.848 and 0.913, Sp= 0.865 and 0.838, respectively) (Table 14). When MAT on cardiac juice is
493 positive on dilutions of 1/6 and 1/12 the chances that bioassay on the heart of the same animal will be
494 positive rise 6.27 and 5.63 times, respectively (Table 14). Results from Table 14 demonstrate that
495 qPCR on heart digest can be used to predict the results of bioassay (Se=0.739 and Sp=0.838) but the
496 other way around is not possible (Se=0.919 and Sp=0.564) (Table 14).

497 The best kappa value was shown between cardiac juice and mouse bioassay (Cohen's kappa=
498 0.66, 95%CI: 0.52-0.80) (Table 14). Results of *T. gondii* antibody detection from sera had fair
499 correlation with bioassay (Cohen's kappa= 0.28, 95% CI: 0.10-0.45) but poor both with qPCR and
500 MC-PCR (Cohen's kappa= 0.20 and 0.05, respectively) (Table 14). When results of any direct
501 detection method were compared to MAT on two matrices moderate kappa agreement was established
502 with cardiac juice (Cohen's kappa= 0.41), while sera showed the poorest concordance (Cohen's
503 kappa= 0.18) (Table 14).

504

505

506 **3.4.4. Concordance between direct detection methods**

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

510

511 **3.5. Discussion.**

512

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

516 (60, 112, 161). In the same time, the best concordances were obtained between MAT with LAT,
517 ELISA, and cELISA (43, 44, 156). Though all contemporary ELISA for pigs showed high sensitivities
518 and specificities when sera were tested, their performance on tissue fluids was lower than MAT (62).
519 In order to obtain tissue fluid from pig carcasses meat or organ samples have to be frozen at -20°C for
520 several hours (24h) (162, 163). The intensity of more than 2000 animals slaughtered per hour in
521 abattoirs (110), does not allow the use of tissue fluids in regular *T. gondii* diagnostics on the slaughter
522 line. Therefore, for this study culled juices from fresh heart samples (mixture of blood and tissue
523 fluids collected without freezing) were compared with sera for detection of anti *T. gondii* antibodies
524 by MAT. This is the first report that compares the MAT performance on different tissue liquids of
525 same animals as well as with direct detection of live parasites and parasitic DNA.

526

527 **3.5.1. Cut off analysis for MAT on sera and cardiac juice**

528 In routine human diagnostic of *T. gondii* infection MAT has been used as described by Desmonts
529 and Remington (42), starting from dilutions 1:20 or 1:25 (164-166). The use of MAT 1:20 dilution in
530 human diagnostic served as a basis for studies of *T. gondii* natural infection in animal studies as well
531 (167-175). But seropositive animal does not always harbour infectious parasites in edible tissues
532 (176). Hence, new studies try to determine the concordance between concentrations of specific *T.*
533 *gondii* antibodies and isolation of infectious parasites in meat (47, 49, 54). The lowest dilution
534 reported in literature – 1:6 was chosen to start the analyses in order to investigate the cut off values for
535 sera and cardiac juices, When sheep cardiac juices are MAT positive in dilutions $\geq 1:16$ isolation of
536 infectious *T. gondii* from heart digest was successful in 65% of cases, while bioassay failed to isolate
537 parasites in 95% of animals when MAT was $<1:16$ (sensitivity= 0.95, specificity=0.59)(54). In this
538 study, ROC analysis set the best cut off for both pig serum and cardiac juice at 1/9, with similar
539 sensitivities (sera=0.86 and 0cardiac juice=0.83), and specificities,(0.71 and 0.85, respectively), as
540 well as moderate predictive ability of the test (AUC=0.888). In pigs MAT cardiac juice positive on
541 dilution 1:12, 79.5% of parasite isolations was successful, while from animals MAT $<1:12$, bioassay
542 was negative in 93.3% of cases. Since MAT is a species-independent serological test, commonly used
543 with animal matrices to detect specific anti - *T. gondii* IgG antibody presence (77), we can conclude
544 that MAT dilution 1/6 is not specific neither for sheep nor for pigs when both sera and cardiac juices
545 are tested. In the same time difference of one third in MAT cut-offs (1/16-sheep and 1/9-pigs) shows
546 higher parasitic burden in pigs than sheep. Nevertheless isolation of one strain MAT negative as well
547 as 2 strains MAT 1/6 positive on cardiac juice (in total 3/140) is acceptable error when considering
548 sensitivity of 79.5%.

549

3.5.2. Comparisons between MAT on sera and cardiac juices

550
551 In 2015 Wallander, Frossling (177) compared detection of specific anti – *T. gondii* antibodies in
552 sera and seven different meat juices of experimentally infected pigs, using the commercial ELISA.
553 Although this study showed that meat juice is not a homogenous serological matrix for diagnostic of
554 *T. gondii* infection, the highest correlation was calculated between heart juice and sera. However,
555 when compared detection of antibodies in heart juice and sera by MAT with isolation of live parasites
556 in bioassay, MAT showed higher sensitivity than ELISA (62). In our study, out of 148 pig samples,
557 antibodies were detected by MAT in 61% of sera and in 39.1% of cardiac juices, while infectious
558 parasites were isolated in 28.6% of bioassays. However, concordances amongst MAT results were low
559 according to the Cohen’s kappa test. Meanwhile Pearson’s product moment correlation test,
560 comparing results obtained on all seven dilutions, showed almost perfect agreement between MAT on
561 sera and cardiac juice (Corr = 0.81; 95%CI: 0.73 – 0.86). Moreover, the existing difference between
562 two matrices is more evident on lower dilutions (1/6, 1/12, 1/25, 1/50), where sera always showed
563 higher concentration of antibodies. The samples for this study were collected at the slaughterhouses
564 from naturally infected pigs of all ages. Therefore, time between infection of animals and their
565 slaughter as well as incubation period was impossible to determine. In pigs, as in other mammals first
566 immune response can be detected during the parasitaemia in blood (178, 179) two weeks after
567 infection (91, 125). If the infection was recent or parasitic load was low, the immune system of certain
568 animals would have a little or no time to develop adequate response to parasites and MAT detectable
569 concentrations of antibodies in all tissues would not be accomplished. Therefore, the presence of anti -
570 *T. gondii* antibodies in heart juice would depend on the blood concentration in the tissue, left after the
571 animal was slaughtered. This means that a large proportion of the discordances might be consequence
572 of low antibody concentrations, low homogeneity of cardiac juice compared to sera and tissue fluids,
573 and insufficient sensitivity of MAT performed on such samples.

3.5.3. Concordance results between direct detection methods

574
575 Out of three direct detection methods, qPCR on heart digest was the most sensitive with 55
576 (42.6%) positive results, followed by mouse bioassay - 37 (28.7%) and MC-PCR on diaphragm - 23
577 (17.8%) positive results (figure 6). For *T. gondii* infection in pigs, brain is the predilection site, but in
578 the absence of this organ, heart is ranked as second in mouse bioassay and third in MC-PCR, while
579 muscle tissue was on fourth position (91, 180, 181). In this study mouse bioassay and qPCR were
580 performed on pig heart digest, while in MC-PCR parasitic DNA was extracted directly from frozen
581 diaphragm tissue, which could influence final concordance results between tests. In the same time,
582 bioassay identifies infectious parasites, while two PCR based techniques target DNA from dead or live
583 parasites. Therefore, the use of two tissues and different objectives for three tests, can explain
584 considerable differences between results. Still the best concordance was observed between bioassay
585 and qPCR confirming the heart as one of the tissues of choice for *T. gondii* analysis in pigs. Compared
586 to bioassay, qPCR failed to detect correctly 16.2% of isolates, showing relatively low sensitivity

587 (0.74) and specificity (0.84). Nevertheless, qPCR identified correctly majority of truly negative
588 samples (NPV=0.918), but claimed high number of false positive samples (PPV=0.564) (qPCR
589 positive, but bioassay negative). Therefore, if the qPCR on pig heart digest is negative there is 4.6
590 times higher likelihood that infectious parasites will not be isolated in bioassay. MC-PCR detected
591 accurately *T. gondii* isolates (sensitivity 0.913), but the number of “false negative” samples resulted in
592 poor specificity (0.405). For this study, diaphragms were frozen for several months, while heart
593 digestion, bioassay and qPCR were performed upon the arrival of fresh samples in the laboratory.
594 Although even a short term freezing of meat has a major impact on *T. gondii* infectivity as proven by
595 Hill, Benedetto (103) and Kotula, Dubey (182), there was no impact on preservation of parasitic DNA
596 (183, 184). Moreover, while pork meat, at -25°C keeps it’s organoleptic characteristics for 12 month
597 because of low activity of present enzymes (185), parasites are concentrated in tissue cysts, which is
598 protected by double membrane. Therefore, the low concordance with both, mouse bioassay (0.36) and
599 qPCR (0.21), as well as low specificity (0.405), positive (0.652) and negative (0.792) predictive values
600 indicate that MC-PCR on pig diaphragm cannot be used in regular diagnostic of *T. gondii*. After
601 experimental infection of 10 pigs with VEG strain (type II), on various muscles 14.1% qPCR and
602 64.6% mouse bioassays was positive (186), while no PCR and 2 mouse bioassays were positive from
603 retail meat in Mexico (152). Contrary, our study showed 47.1% qPCR and 29.2% of mouse bioassay
604 positives when analysis was performed on the same digested hearts, while 34 pigs were both positive
605 on both techniques (51.5% and 82.9%, respectively). Hears for both bioassay and qPCR were prepared
606 as described in (187, 188), with the goal to liberate bradyzoites from tissue cysts and obtain
607 homogenous number of parasites in the sample (108). Since for the success of bioassay is important
608 that there is infectious dose of live parasites and qPCR results depend upon the presence of parasitic
609 DNA (extracted from live or dead parasites in tissue), one of the explanations is that high numbers of
610 *T. gondii* harboured in pig hearts were dead.

611 **3.5.4. Comparison between indirect and direct detection methods**

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

623 the other hand MAT on sera correctly detected truly negative animals (NPV 0.841) but failed to detect
624 around 50% of positive pigs (PPV of sera: 0.46 – dilution 1/6, 0.58 - dilution 1/12, and 0.64 - dilution
625 1/25). MAT on cardiac juice had better sensitivity and specificity when results of qPCR on heart
626 digest or MC-PCR on diaphragm were used as references. Based on these results the MAT on cardiac
627 juice is considered as “reference” matrix which can be used as a screening test.

628 **3.6. Conclusions**

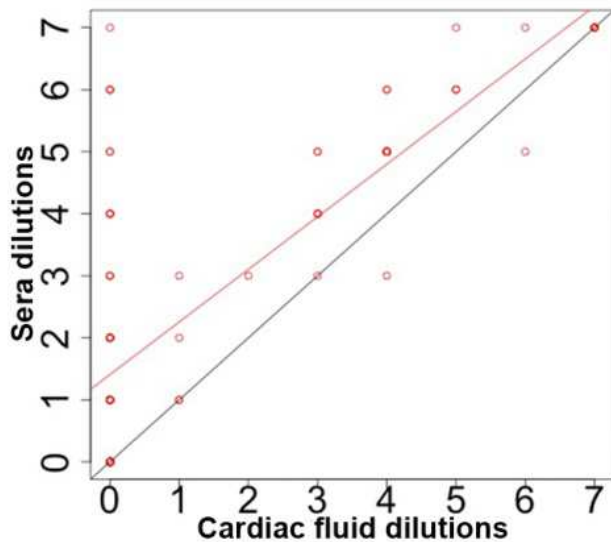
629 Still, one strain was isolated by mouse bioassay from one pig that was negative in both
630 indirect detection methods (MAT on sera and cardiac fluid) and direct detection method of qPCR.
631 Considering sensitivity and positive predictive values of both matrices (sera PPV=0.456; cardiac fluid
632 PPV=0.696; and diaphragm fluid PPV=0.378) as well as qPCR (PPV=0.564) the error of only one
633 strain is acceptable for MAT and detection of specific DNA. A lack of MAT specificity with cut-off
634 1/6 can be overcome if the test limit is set to 1/9, as indicated by ROC analysis. However, when using
635 a higher cut-off value, 2 bioassay positive strains would have been assigned to pigs with serological
636 results below the threshold limit (2 isolated strains from pigs with a 1/6), and the meat of these
637 animals would arrive on the public markets as *Toxoplasma*-free.

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

653

654 **Figure 4 Correlation of MAT on sera and cardiac meat juice in 7 dilutions (1/6, 1/12, 1/25, 1/50,**
 655 **1/100, 1/200, >1/200).**

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

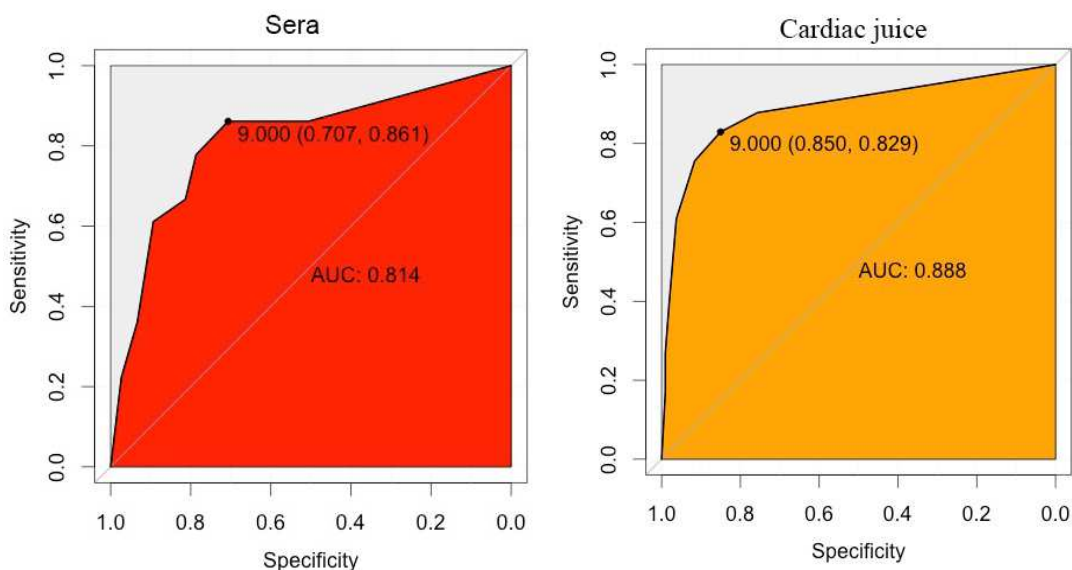


661

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

664 The ROC analysis shows that the highest sensitivities and specificities that can be achieved using
 665 MAT on any tissue fluid are Se=0.850 (cardiac meat juice) and Sp=0.861 (sera). Nevertheless separate
 666 ROC analysis on both pork matrices rappsorts the ideal cu off value of 1/9, with highest sensitivity and
 667 specificity for cardiac fluid (Se=0.850, Sp=0.829), and high probability value (AUC = 0.888).
 668

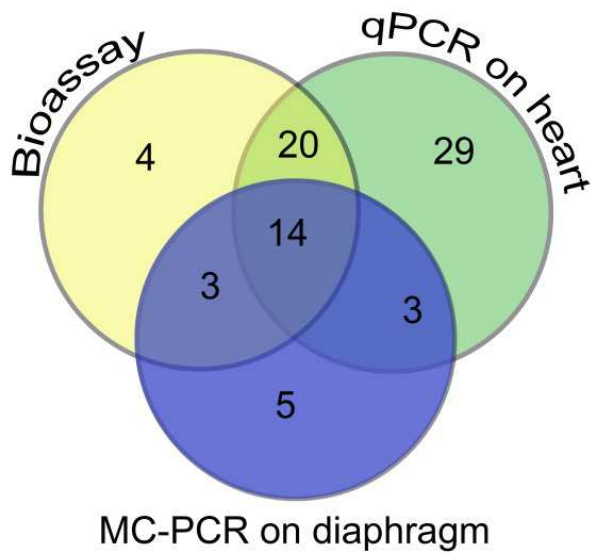
668



669

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

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



672

673

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

676 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																	684
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																	689
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	

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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-PCR0.913	0.913	0.405	0.65%	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			

719

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722 **4. Section 4**

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

725

726

727 **4.1. Summary**

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

753

754 4.2. Introduction:

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

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

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

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

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

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

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

809

810 **4.3. Materials and Methods:**

811

812 **4.3.1. Antigen definition:**

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

815

816 **4.3.2. Mouse antigen preparation:**

817 Mouse antigen was prepared according to Desmonts and Remington (1980) (Table 16). In short
818 2×10^6 *T. gondii* tachyzoites of Rh strain were inoculated i.p. into each female Swiss Webster CD I
819 mouse (4 weeks old) with 5×10^4 TG 180 sarcoma cells. After two days, mice were sacrificed and
820 intraperitoneal exudate was harvested, mixed with new 2×10^4 sarcoma cells (obtained from mice
821 infected only with these cells, without parasites). Forty-eight to 72 hours later intraperitoneal fluid was
822 collected, centrifuged and digested in trypsin (Table 16). Digested cells and liberated parasites were

823 washed in PBS and centrifuged after which fixation in formalin was done for 16-20 hours. Fixed
824 parasites were washed three times in PBS, counted and diluted in alkaline buffer in concentration of
825 2×10^8 / ml (Table 16). Sodium-Azid buffer was added to store the antigen.

826

827 **4.3.3. Cell culture antigen formulation:**

828 Three different cell cultures were tested separately in this experiment. Flasks of 75ml with: i)
829 human foreskin fibroblast (HFF) cells, ii) human colon ileocecal colorectal adenocarcinoma (Hct-8)
830 and iii) human epithelial colorectal adenocarcinoma cells (Caco-2) were prepared independently
831 according to Wu et al (2009). When reached 95% of confluence, cell flasks were inoculated with $2 \times$
832 10^6 Toxo Rh strain tachyzoites. Type I, Rh strain is fast replicating strain in almost any type of tissue
833 culture, cloning at the rate of $\approx 119/48h$. After 48 - 96 hours parasites were harvested from flasks and
834 antigen was prepared according to the procedure described in Table 16. The protocol, established by
835 Desmonts and Remington, was improved by replacing the mice with cell cultures for amplification
836 and preparation of *T. gondii* Rh tachyzoites. In brief, $2 - 5.5 \times 10^7$ of parasites/ml was collected from
837 flasks two days after inoculation, when 95-100% of cells were destroyed. Tachyzoites were washed
838 twice in PBS in order to eliminate cell debris, cell medium leftovers and broken parasites. Fixation
839 was then done for minimum 16 hours in formaldehyde. Two washings before and after fixation in PBS
840 were determined as optimum limiting parasite loss. Fixed tachyzoites were counted and diluted in
841 alkaline antigen buffer in adjusted concentration of 2×10^8 per ml (Table 16).

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

845

846 **4.3.4. Samples for testing**

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

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

859

860 **4.3.5. Modified agglutination test**

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

869

870 **4.3.6. Statistical analysis**

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

880

881

882 **4.4. Results:**

883

884 **4.4.1. Antigen production**

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

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

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

896

897 **4.4.2. Concordance:**

898 The Cohen's kappa showed excellent concordance between antigens produced from mice and the
899 one produced from HFF cultured parasites (Cohen's kappa between 0.64 - 1) (Table 17). The lowest
900 concordance between two antigens was observed when pork sera were tested (Cohen's kappa
901 substantial - 0.64, 95%CI: 0.4-0.9). Almost perfect concordance was shown on goat, cat sera, and
902 bovine cardiac fluid since mouse antigen yielded higher numbers of seropositive animals (Cohen's
903 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
904 17). Dog sera and all controls, demonstrated perfect concordances between antigens produced in mice
905 or in cell culture (Cohen's kappa 1.00, 1.00, 1.00, respectively). McNamara test validated statistically
906 substantial similarities between antigens for all samples (McNamara p-value between 0.074-NA),
907 except for pig sera (p-value: 0.013). Highest differences are observed on pork samples but still not
908 statistically significant (Table 17).

909

910 **4.4.3. Correlation:**

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

919

920 **4.4.4. Cost-benefit:**

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

924 **4.5. Discussion**
925

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

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

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

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



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

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

984

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

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

Procedure		A) Mouse antigen production 	B) Cell culture antigen production 
1	Inoculation with 2*10 ⁶ parasites	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
2	Inoculation with 5*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*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, H3BO3, 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"/>

993 *Parasites should be collected 2-3 days after infection in 3rd or 4th stadium when half of the parasites
 994 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)
163	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 patientes	(205)
1995	HFF	Bradyzoite developement 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 carcase. 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

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

39

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

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

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

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

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

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

97

98 **Quantitative prediction of pork as an important source for human infection**

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

104 As described previously, results from nation-wide studies of three major production species
105 (pigs, sheep, cattle) support the fact that seropositive animal is no automatically carrier of infectious *T.*
106 *gondii*. In pig study from intensive farms out of 963 fattening pigs, in 22 cases specific anti - *T. gondii*

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

113 French abattoirs in 2103 slaughtered 24,396,000 pigs out of which 4,915,000 were piglets (up
114 to 2 month of age or 25kg weight (Agreste., 2015). On a domestic market in France nearly 2.1×10^6
115 tones of pork are sold, divided between 1.2×10^5 tones of piglet meat and 1.9×10^6 tones of fattening and
116 reproduction animals pork. Out of these numbers 98.5% of pigs were grown in intensive farms while
117 only 1.5% originated from outdoor farms. In nation-wide study no *T. gondii* strain was isolated from
118 piglets, therefore further analysis will concentrate on fattening pigs and sows. The same study found
119 adjusted 2.9% prevalence of *T. gondii* in 5,337,193 intensively bred fattening pigs, and 13.4% in
120 13,851,592 sows. This means that 154,779 fattening and 1,856,113 reproduction animals have
121 detectable concentrations of anti – *T. gondii* antibodies. When sensitivity of bioassay (0.5) is taken
122 into account, we can calculate a total of 1005446 intensively bred pigs in France that harbour *T. gondii*
123 parasites. Average weight of fattening pigs at the abattoir is 115kg (110-140kg), from which animal
124 carcass after slaughter weights around 86kg. From obtained meat 25-30kg are cut for different
125 produces and 55-60kg are sold as fresh pork. This means that on French markets 86,468 tons of pork
126 infected with *T. gondii* was sold. Hence out of total intensively bred pork, consumed in France, 4.1%
127 harboured infectious *T. gondii* parasites. In the same time, 1.5% of French pork market consists of
128 meat from outdoor-bred animals. Nation-wide study found 6.3% adjusted prevalence for 101,028
129 fattening pigs from outdoor farms. Calculated sensitivity of bioassay in outdoor pigs is 0.94, which
130 computes 5983 animals that harbour infectious *T. gondii*. From 5983 animals 514.5 tones of meat was
131 sold to a consumers. Therefore, out of total number of outdoor raised pigs destined for human
132 consumption, 5.9% were infected with live *T. gondii* tissue cysts. This proves that consumption of
133 pork originating from outdoor farms exposes humans in higher extent to *T. gondii* infection. However,
134 pork from intensive farms is more represented in French cuisines, and infected pieces count for 4.1%
135 of all pig meat consumed in country. Whereas infected meat from outdoor-bred animals, accounts only
136 for 0.2% of total meat consumed in France. In 2013, France had 65525000 habitants (244), and pork
137 consumption was 33.5 kg/capita (Faostat03, 2015). Therefore, there is 20.4 times higher probability
138 that humans in France were in contact with pork contaminated with *T. gondii*, originating from indoor
139 than from outdoor farms.

140 Nevertheless, this calculation does not mean that there is 20 times greater chance of acquiring
141 *T. gondii* infection if pork from intensive farms is consumed in contrast to outdoor raised animals. For
142 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,

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

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

181

182 **Prevalence of *T. gondii* in intensive pig farms from Bretagne: comparison between two** 183 **studies**

184

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

200 It may seem that overall *T. gondii* prevalence of 6.9% in intensively bred pigs in Bretagne
201 region of France is higher than one calculated in Nation-wide study in 2013. For the risk factor
202 analysis and prevalence in intensively bred pigs in Bretagne, farms were randomly chosen based on
203 the annual numbers of animals (finally 500 suckling piglets were sampled, 389 weaned piglets, and
204 2532 fattening pigs). Whereas for French national study, regions were chosen based on the annual
205 numbers of slaughtered pigs and each was represented with the same number of samples (Bretagne
206 with 35 piglets, 150 fattening pigs, 70 sows samples from intensive farms). From pigs on intensively
207 bred farms examiners collected sera directly. For nation-wide study heart and blood samples were
208 collected by abattoir veterinarians. In order to avoid possibility that heart and sera samples were
209 mismatched in national study, cardiac fluids were used for prevalence analysis. In the same time, 37%
210 of sera were missing from Bretagne, therefore seroprevalence could be calculated for 35 piglets, 61
211 fattening pigs and 64 sows (total=160 animals). Straight a way it is evident that Bretagne is under

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

226 When prevalence for fattening animals was calculated (only category in common), 6.4%
227 *Toxoplasma* seropositive pigs were found in Bretagne study in contrast to 1.6% seropositive and 3.3%
228 of cardiac fluid positives in national study. If two piglet categories from Bretagne survey would be
229 merged together, obtained seroprevalence is 8.5% in contrast to 0%; and 2.9% prevalence of cardiac
230 fluid in national study (Table 20). One of the explanations can be low number of samples from
231 Bretagne collected in nation-wide survey. When prevalence is low, in order to confirm it, the number
232 of collected samples should be higher (Thrusfield., 2007), otherwise standard error becomes non
233 acceptable. When the number of samples is low as intensive pig sera in nation-wide study, confidence
234 intervals are considerably higher. When compared 95% confidence interval extremes, results in two
235 studies are more similar. The sera for study of *T. gondii* in pigs from Bretagne were collected from
236 2006 – 2008, while collection of samples for national study was done in 2013. One of the possibilities
237 is that as well as in other countries, by improving the pig breeding methodology, zoo-technical and –
238 hygienic conditions on farm, infection rate dropped in just several years (145), which would make
239 obtained results comparable.

240

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

243

	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	0.000	0.016	0.041
95% Confidence interval	4.8 – 9.1	7.4 – 13.6	6.7 – 10.3	5.4 – 7.4		-1.5 – 4.7	4.5 – 20.5

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245

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247

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

250

251 Results from literature about seroprevalence analyses in production animal (Tables 3 and 4)
252 show extensive *T. gondii* infection of all domestic species. Sheep are the most infected, with
253 prevalence ranging from 15% in lambs from France and 27.8% in adults from the Netherlands, to
254 84.5% in Serbia and 89% in France. Sheep are bred mainly on pastures, which makes them the most
255 exposed to environmental contamination with *T. gondii* oocysts. Even if kept inside (during winter
256 months), sheep are fed with dried hay and little or none of other food is used. In contrast to sheep
257 nutrition, dairy cows and lactating goats besides pasture and hay are fed with concentrated nutrients.
258 This can explain slightly lower prevalence of *T. gondii* in these two species in contrast to sheep.
259 Lowest prevalence in goats was recorded in Portugal (18.5%) while highest infection rates were found
260 in Serbia (73.3%). In cattle, lowest seroprevalence was found in Portugal as in goats (7.5%) while
261 highest were reported in Spain (83.3%)(Table 3). While seroprevalence in all three ruminant species
262 vary greatly throughout Europe, it is still discussed if the meat, meat products or milk from these
263 animals represents danger for public health. Meat from sheep and goats as well as their delicatessen
264 are well treated in salts or well boiled in order to make it tenderer, while the lamb or goat kid meat in
265 some countries is prepared half cooked (“agneau rose” in France for example). Until today, only one
266 study isolated live *T. gondii* from clinically healthy cattle in nation-wide study in France, when heart
267 samples were collected on the slaughter line (11). In the same time, numerous studies report about
268 isolation of the parasites from milk and milk products of all ruminants, especially goats (256-261).
269 From literature data about *T. gondii* occurrence in ruminants we can only hypothesise that infection
270 extent is directly related to breeding methods and levels of its development from country to country. In
271 Spain for example, cattle are ranched in semi extensive conditions and most of the time animals spend
272 on pastures, same as goats. In the same time in ex Yugoslavian countries goats were forbidden species
273 for over 30 years, and animals have been imported in the last 10 years in Serbia, and seroprevalence is
274 high as a result of low developed breeding methods. In Europe, equine infection with *T. gondii* is
275 reported in 5 countries. The lowest seroprevalence was recorded in horses from Sweden, while highest
276 intensity of infection was found in horses from Romania (Table 3). When considered that only in
277 France, Switzerland, Italy and Japan horsemeat is eaten raw or semi cooked the risk of acquiring
278 infection can be reduced to these countries. Isolation of infectious *T. gondii* was successful from
279 experimentally infected animals, but data about naturally infected animals are scarce and not well
280 studied.

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

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

309

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. Desmots 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.

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List of Publications

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714 Scientific articles:

- 715 1. **Djokic V.**, Blaga R., Aubert D., Durand B., Perret C., Geers R., Ducry T., Vallee I., Djurkovic
716 Djakovic O., Mzabi A., Villena I., Boireau P., 2015. *Toxoplasma gondii* infection in pork
717 produced in France. Parasitology – accepted article
- 718 2. Radu Blaga, Dominique Aubert, Catherine Perret, Régine Geers, **Vitomir Djokic**, Isabelle
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742 Viorica Mircean, Anamaria Ioana Paștiu, Anamaria Balea, Zsuzsa Kalmar, Diana
743 Bărburaș, Edoardo Pozio, Furio Spano, Georgina Limon, Milen Georgiev, Damer Blake,
744 Javier Guitian, Javier Dominguez, Frank Katzer, Alison Burrells, Lee Innes, Olgica
745 Djurkovic-Djakovic, Ivana Klun – EFSA **External scientific Report: Relationship**

746 **between seroprevalence in the main livestock species and presence of *Toxoplasma***
747 ***gondii* in meat (GP/EFSA/BIOHAZ/2013/01) - An extensive literature review**

- 748 2. Marieke Opsteegh, Gereon Schares, Radu Blaga, Joke van der Giessen and consortium:
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753 Edoardo Pozio, Furio Spano, Simona Cherchi, Georgina Limon, Milen Georgiev, Damer
754 Blake, Javier Guitian, Javier Dominguez, Frank Katzer, Alison Burrells, Lee Innes, Olgica
755 Djurkovic-Djakovic, Ivana Klun - **EFSA External scientific Report: Experimental**
756 **studies on *Toxoplasma gondii* in the main livestock species**
757 **(GP/EFSA/BIOHAZ/2013/01)**
- 758 3. Radu Blaga, Catherine Perret, **Vitomir Djokic**, Tamara Ducry, Isabelle Vallée, Pascal
759 Boireau, Dominique Aubert, Régine Geers, Isabelle Villena, Benoit Durand – **DGAL :**
760 **Contamination par *Toxoplasma gondii* des viandes de boucherie (espèce porcine)**
761 **Résultats du plan de surveillance 2013**

762

763 **Oral Presentations on international congresses:**

- 764 1. **V.Djokic**, G. Limon, C.Perret Duménil, D.Aubert, R.Geers, T.Ducry, B.Durand, P.Boireau,
765 I.Villena, J. Guitian, M. Georgiev, J.Dominguez, J. van der Giessen, R.Blaga,. *Toxoplasma*
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