Toxoplasma gondii: pig seroprevalence, associated risk factors and viability in fresh pork meat

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Abstract

This study was conducted on 161 fattening pig farms located in Aragón (Northeast Spain). Serum samples from 1,200 pigs were tested for antibodies against *T. gondii* by indirect immunofluorescence assay (IFA). Antibodies to *T. gondii* (≥1:20) were detected in 301 pigs (24.52%). The seroprevalence observed in the present study indicates a widespread exposure to *T. gondii*, as seropositive pigs were found in 96.67% of the farms studied although low pig titers were determined. Risk factors associated with *T. gondii* seroprevalence were presence of cats in or around the farms, presence of dogs

around the facilities, low number of animals in the farms, poor hygiene and bad maintenance of the farms. Finally, it was observed that where rodent baits were used, *Toxoplasma* prevalence was lower. Risk management measures including control of cats and rodents on the farms, among others, could help to reduce the observed prevalence levels. By mouse bioassay, *T. gondii* was detected in 73.7% and isolated from 42.1% of seropositive pigs and a significant relation between the titers of pigs and the presence and viability of *T. gondii* in the tissues was found. The detection of *T. gondii* is not possible by currently practiced meat inspection. Nevertheless, the increased probability of detecting viable forms of *T. gondii* in tissues of pigs with titers ≥1: 80 could be used as the cutoff for discriminating higher risk animals, and could be used as an effective control tool for the industry of cured meat products. In practical terms, we propose that this value could be used as a critical limit in the HACCP system.

Keywords

Toxoplasma gondii; pig seroprevalence; risk factors; pork meat; safety control tool; HACCP; cured meat products

Introduction

Toxoplasma gondii is one of the most successful protozoan parasites in nature, being able to infect all warm-blooded animals including humans. In most adults, infection does not cause serious illness, but severe disease may occur in immunocompromised people. In pregnant women who become acutely infected, the parasite can also cause

severe abnormality or death to the unborn child (Tenter et al., 2000; Hill et al., 2005; EFSA, 2011).

Although toxoplasmosis is conventionally associated with cats and kitty litter, it is estimated that 50% of cases are foodborne, and the USA CDC (Centers for Disease Control and Prevention) estimate that foodborne toxoplasmosis is surpassed only by *Salmonella* in the number of annual deaths it causes (Scallan et al., 2011). Further, from an international food safety perspective, *Toxoplasma* ranked 4th among foodborne parasites with the greatest global impact (FAO/WHO, 2014). Specifically, consumption of raw, undercooked or cured meat products containing tissue cysts is the major risk factor associated with human toxoplasmosis (Kapperud et al., 1996; Baril et al., 1999; Cook et al., 2000; Berger et al., 2009). Due to its omnivorous character, pigs have great possibilities of acquiring infection with *T. gondii*, being the consumption of pork meat one of the most important sources of infection (García-Bocanegra et al., 2011; Balea et al., 2012; Juránková et al, 2014).

The seroprevalence of *T. gondii* in pigs is highly variable among countries and regions within the same country, and is influenced by the production system. Studies in different countries indicate a seroprevalence from 0.4% to 90.4% (Dubey, 2009; Guo et al., 2015).

The risk of detecting antibodies to *Toxoplasma* in extensive production farm pigs is statistically higher than in industrial farm pigs, and prevalence varies dramatically among the classes of pigs surveyed (market pigs *versus* sows) (Tenter et al., 2000; Dubey and Jones, 2008; Dubey, 2009; Alvarado-Esquivel et al., 2011; Yu et al., 2011;

Guo et al., 2015). Production system is an important factor in the process of risk assessment, taking into account current trends in the consumption of meat from organic farms that produce their pigs in extensive, which could cause a resurgence of infection rates (Van der Giessen et al., 2007; Dubey, 2010; García-Bocanegra et al., 2010a; Veronesi et al., 2011). Besides this, one of the most important risk factors that enables contact with the parasite in pig farms is the presence of cats (García-Bocanegra et al., 2010a, 2010b; Ortega-Pacheco et al., 2011; Cenci-Goga et al., 2013). Other factors that contribute to infection of pigs are the age of the animals and the size of the census, the facilities of the farms and their maintenance, rodent control to avoid that they can be eaten by pigs, the possibility of contact with dead bodies, or a favorable temperature for oocysts sporulation (Villari et al., 2009; García Bocanegra et al., 2010b; Hill et al., 2010; Veronesi et al., 2011; Cenci-Goga et al., 2013; Hernández et al., 2013).

Seropositivity is, in general, a good indicator of the presence of the parasite in tissues (Dubey et al., 1995a, 2002; Dubey and Jones, 2008) and some authors report that the level of isolation increases with antibody titer in the pig (Dubey et al., 1995b). *T. gondii* shows high affinity for neural and muscular tissues (Dubey, 2009), and a worldwide prevalence in carcasses of pigs for human consumption, ranging from 0.4% to 92.7%, has been reported (Dubey, 2009; Guo et al., 2015). *Toxoplasma* cysts in pork can persist for a long time (EFSA, 2007). However, pork meat enters the food chain without a specific meat inspection to check *T. gondii* in the slaughterhouse (Dorny et al., 2009; Blagojevic and Antic, 2014), identified as one of the most relevant biological hazards in the context of meat inspection of swine (EFSA, 2011).

Spain is the fourth largest producer potency of pork after China, USA and Germany (MAGRAMA, 2014), and meat industry has focused the attention to produce meat that is wholesome, safe, and of high quality. Dry-cured ham is an important food in the Mediterranean area, and Spain is one of the main producers. This type of ham is widely consumed in Spain and exported to other countries, so the prevalence at farm level should be reduced to produce uncontaminated raw materials and elaborate safer hams. To ensure food safety throughout the food chain, preventive measures should be focused in primary production (e.g. surveillance and monitoring of animals), and post-harvest strategies at slaughter and during food processing. At farm level, risk reduction measures are based on herd health programs, closed breeding pyramids and GHP/GFP. The application of hazard analysis and critical control point (HACCP) principles to primary production is not yet generally feasible. However, guides to good practice should encourage the use of appropriate hygiene practices at farm level (Commission Regulation No 852/2004; EFSA, 2011).

Considering its importance for the risk assessment process, the aim of this work is to know the prevalence of *T. gondii* antibodies in finishing swine raised in different farms of Aragón (Northeastern Spain), as well as to evaluate the risk factors for the transmission of the parasite at farm level for determining the best management practices to reduce the potential of infection in order to obtain "*Toxoplasma* Free Farms". Additionally, this study aims to determine the presence and viability of *T. gondii* in tissues of seropositive pigs, and to evaluate if serological titers could be useful to potentially identify contaminated pork meat destined to elaborate cured meat products.

Materials and methods

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125 *1. Sampling of farms and animals*

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127 The study was conducted on 161 fattening pig farms located in Aragón (Northeastern
128 Spain). For sampling farms and animals within each farm the statistical program
129 WinEpi (Working in Epidemiology) was used to ensure a 95% probability of detecting
130 at least one positive animal for an expected seroprevalence of 16%, which is the
131 prevalence obtained in previous studies in our laboratory and in the scientific literature
132 in Spain. Based on these calculations, a total of 60 pig farms were studied (Figure 1)
133 and 20 pigs were sampled from each farm (50% males and 50% females, between 4 and
134 5 months of age). In total 1,200 fattening pigs were analyzed. Specifical identification
135 of pigs was carried out for traceability of pig sera.

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137 2. *Study of seroprevalence of T.gondii in pigs*

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139 *2.1. Serum sampling*

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141 Three milliliters of blood were obtained by puncture of the jugular vein into sterile 5-ml
142 tubes (BD Vacutainer, no additive, BD, Franklin Lakes, NJ). The serum was obtained
143 by blood centrifugation at 3,500 rpm for 10 min (Hettich Universal, Germany),
144 transferred to 1.5 ml eppendorf tubes and stored at −20 °C until analyzed.

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146 *2.2. Serological examination*

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Pig sera were analyzed by indirect immunofluorescence assay (IFA) (bioMérieux, Marcy l'Etoile, France) to detect antibodies against *T. gondii*. Serum dilutions of 1:20, 1:40, 1:80, 1:160, and 1:320 were prepared from each sample to be tested. A positive control (kindly provided by Dr. J.P. Dubey), and a contrasted negative control were included in each analysis. All prepared slides were examined with an Eclipse 80i fluorescence microscope (Eclipse 80i, Nikon instruments INC, Netherlands). A positive result was determined when clear whole-perimeter tachyzoite fluorescence was observed, and the cutoff for positivity was 1:20.

3. Assessment of risk factors in farms

Epidemiological data were collected from 161 farms through an on-farm interview of the farmer and/or the veterinary based on "closed-ended" questions, and also through visits to the farms. Data obtained were contrasted with pig serological results from the sampled farms. The following variables were included to provide information on exposure levels to potential risk factors:

- General data of the farm: identification, location and number of animals.
- Production parameters and behavior: sex of the animal, production system and cannibalism.
- Facilities: outdoor facilities and floor type (fully slatted, partially slatted).
- Conservation and maintenance of farms: use of exclusive clothing, use of hot water and soap to clean, presence of weeds around the farm, window status and temperature maintenance system.
- Presence of animals inside or outside the farm: cats, dogs, rodents, birds and others.

- Rodent control: baits, cats or specialized companies.
- Feeding conditions: feed administration (wet, dry), water source (river, irrigation ditch, or well) and water treatment.

4. Analysis of pig tissues for T. gondii

Forty one Pietrain x Landrace pigs were selected for this study: 3 animals with a titer <1:20, as negative controls, and 38 seropositive pigs, with different titers (5 with a titer 1:20, 9 with 1:40, 16 with 1:80 and 8 with 1:160). The selected pigs were slaughtered in a commercial slaughterhouse. Tissues selected for analysis were raw ham, heart and tongue. The pig hams were boneless, sliced into small pieces, and minced. Tongue and heart of each pig were also minced, and both organs were mixed at 50%. The minced tissues were completely homogenized, vacuum packaged and stored at refrigeration until analyzed. Specifical identification of pig tissues with corresponding pig sera was carried out for traceability.

4.1. Mouse bioassay of tissues for T. gondii

A concentration bioassay technique with the acid pepsin digestion procedure was used to demonstrate viable bradyzoites of *T. gondii* in tissues, as previously described (Dubey, 1998; Bayarri et al., 2010). A 0.5-ml aliquot of digestion extract was inoculated intraperitoneally into each of eight 20-25 g CD1 Swiss female mice (Janvier Labs, Le Genest-Saint-Isle, France). All experiments included negative control mice, which were analyzed at the end of the process. Possible disease symptoms were monitored in

197 inoculated mice and animals showing distress signs were euthanized as an animal
198 welfare measure.
199
200 Blood samples were drawn from mice that survived 60 days after inoculation. Samples
201 were centrifuged at 3,500 rpm for 10 min (Hettich Universal, Germany). Sera were
202 transferred to 0.5 ml eppendorf tubes and stored at -20°C until analysis. Subsequently
203 mice were sacrificed in a CO_2 chamber and the brains of each animal were removed and
204 introduced in a 5.0 ml eppendorf tube and stored at -20°C to assess the viability by
205 PCR.
206
207 Mice were maintained at the *Centro de Investigación Biomédica de Aragón (CIBA)*, in
208 Zaragoza (Spain) under conditions that complied with international rules of good
209 laboratory practices in the care of experimental animals (Directive 2010/63/EU). All
210 procedures were approved by the Ethics Advisory Commission for Animal
211 Experimentation and by Biosecurity Commission of the University of Zaragoza
212 (PI07/12).
213
214 *4.1.1. IFA of mouse sera*
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216 To demonstrate the presence of the parasite, sera samples of mice were analyzed by IFA
217 to detect antibodies against *T. gondii* with polyclonal rabbit anti-mouse
218 immunoglobulins (DakoCytomation). Serum from each mouse was diluted 1:10, 1:20,
219 1:40, 1:80, 1:160, and 1:320. A positive control serum provided by the Unit of
220 Pathological Anatomy of the Department of Animal Health (Faculty of Veterinary
221 Medicine, University of Zaragoza) and a checked negative control from previous studies

in our laboratory were included in each test. Final preparations were examined with an Eclipse 80i fluorescence microscope (Eclipse 80i, Nikon instruments INC, Netherlands). Sera samples with a titer ≥1:10 were considered positive.

4.1.2. Analysis of T. gondii DNA from mice brains

To assess the viability of the parasite, 15 mg brain samples of serologically positive mice were analyzed by real time-PCR. DNA extraction from each sample was performed using UltraClean® Tissue & Cells DNA Isolation Kit Sample Catalog No. 12334-S (Mobio Laboratories, Inc.) according to the manufacturer's instructions. DNA amplification targeting specific sequence of 529 repeat element and SAG genes were performed. CFX Connect (Bio-Rad Laboratories) real time PCR instrument was used for the amplification and detection of *T. gondii*. The reactions volume was 20 µl and samples were run in triplicates. The procedure consisted of 7 minutes at 94°C for enzyme activation (hot start), and 40 cycles of denaturation at 94°C for 5 s, annealing at 55°C for 30 s and extension at 72°C for 10 s. The program ended with a dissociation curve from 60 to 94ºC with a 0.5 ºC increase interval. Each PCR run included a negative control, a positive control, and a separate reaction for Actin DNA copies as internal control (lC). A sample was considered positive if at least two of the triplicates were positives with a Ct lower than 35.

5. Statistical analysis

The estimated prevalence of antibodies against *T. gondii* was calculated from the ratio of positive results to the total number of pigs examined, with 95% confidence intervals.

Data about farm conditions and serological results were entered into a database created with the program Microsoft Access 2010. Serological results were compared with the variables studied in the farms to assess the factors associated with *T. gondii* in primary production using Pearson's Chi-square test (or Fisher's exact test when Pearson's Chi-square test was not valid).

Pearson's Chi-square test (or Likelihood Ratio test when Pearson's Chi-square test was not valid) was also used to establish a statistical relationship between the serological titer of pigs and the presence and viability of *T. gondii* cysts in tissues.

Statistical analysis was performed with IBM SPSS 19.0 for Windows. Differences were considered statistically significant when $p < 0.05$.

Results

Seropositive pigs (IFA 1:20 or higher) were found in the 96.67% of the evaluated farms, with a maximum of 13 positive animals in 65.0% of farms and a minimum of 1 positive pig in 5.0% of farms. In the 82.76% of the positive farms, serological titers were ≤1:40. Animals with a titer 1:80 were detected in the 17.24% of the positive farms, while animals with titers 1:160 were detected only in the 6.89% of the farms. No animals with a serological titer higher than 1:160 were found.

Antibodies against *T. gondii* were detected in 301 of 1,200 pigs tested, and seroprevalence was calculated to be 24.52%. In general, low pig titers were determined, as shown in Figure 2.

Risk factors associated to *T. gondii* seroprevalence in the studied farms are shown in Table 1. The presence of cats in or around the farms was the main factor that increases the prevalence of *T. gondii* (p=0.001). The presence of other animals, such as dogs, around the facilities was evidenced as another risk factor (p=0.003). Higher seroprevalence of *T. gondii* infection in pigs was significantly related to a low number of animals in the farms (p=0.009). Related to maintenance and conservation of the farms, poor hygiene (P=0.017) and bad maintenance of the farms (p=0.019) were risk factors with statistical significance. Finally, concerning the method of rodent control, it was observed that using baits (p=0.009) decreased the prevalence.

Presence and viability of *T. gondii* in tissues are shown in Table 2. *T. gondii* was detected in tissues of 28 of the 38 seropositive pigs (73.7%). No cysts were detected in tissues of seronegative pigs. Positive bioassays came from 2 pigs with a titer 1:20, 3 with a titer 1:40, 15 with a titer 1:80 and 8 with a titer 1:160. There was a significant dependence between the titers of pigs and the presence of *Toxoplasma* in tissues ($p < 0.001$). Pigs with a serological titer ≥1:80 have significant possibility to contain tissue cysts. In fact, the percentage of pigs with titers ≥1:80 and tissue cysts was 95.8%.

Regarding viability of *T. gondii* in fresh tissues, real time-PCR positivity in mice brains showed viability of the parasite in 16 of the 38 pigs (42.1%) (57.1% of tissues in which the parasite was detected). There was also a significant dependence between the titers of

pigs and the viability of the parasite (p=0.003). Pigs with a serological titer ≥1:80 have a significant probability to host viable parasites in their tissues. Tissues of 62.5% of pigs with titers ≥1:80 have viable parasites *versus* 7.1% of pigs with titers < 1:80 containing viable parasites in their tissues.

Discussion

The results of this study indicate that *T. gondii* infection is widespread, with up to 96.7% positive farms. However, in general, low titers of pig sera were determined, and wide variations in within-farm seroprevalence were observed, as it has been reported in previous studies (Tenter et al., 2000; Dubey and Jones, 2008; García-Bocanegra et al. 2010b). Similarly to us, surveys based on the presence of antibodies in blood sera have reported a worldwide distribution of *T. gondii* (Dubey, 2010; Guo et al., 2015). The 24.5% prevalence for *T. gondii* found in our study was similar to the prevalence found in others Spanish regions such as Catalonia (21.2%), Extremadura (23.3%), and Valencia Community (27.3%), and higher than data previously obtained in Aragón (10.1-15%) (García-Bocanegra et al., 2010b). Similar seroprevalences were also found in other European countries, such as Ireland (23.1%) and Serbia (28.9%) (Hálová et al., 2013; Klun et al., 2006). Nevertheless, a higher prevalence (43.1%) has been reported in recent studies carried out in Romania (Balea et al., 2012), and lower seroprevalences, from 4.2% to 18.5%, have been reported in Germany (Damriyasa et al., 2004), Italia (Veronesi et al., 2011), Latvia (Deksne and Kirjušina, 2013) and Portugal (Lopes et al., 2013; Esteves et al., 2014). A close comparison among studies is difficult due to the different serological tests used and the different cutoff values that not always are reported by authors. Besides, the lack of validation is shown in the literature by the use

of different cutoffs for a single test, without any data on sensitivity, specificity, and agreement among tests (Aroussi et al., 2015).

Prevalence of *T. gondii* also varied depending on the type of management practices used in the farms, the number of animals tested, the age and type of the pigs tested (fattening *vs.* sows; indoor pigs *vs.* organic pigs) (Dubey, 2009; Guo et al., 2015). Prevalence of *T. gondii* infection in pigs are usually higher in older pigs and pigs reared outdoors than in piglets and pigs on factory farms, because they have a higher probability of contact with infective oocysts or infected intermediate hosts (García-Bocanegra et al., 2010b; Dubey, 2010; Blagojevic and Antic, 2014; Basso et al., 2015).

Data provided by the present study show that the main risk factors in farms were the presence of animals (cats and dogs), size of the farm, cleaning conditions and rodent control. The presence of cats in and out of the farm is shown as the most significant risk factor in this study in agreement with those previously reported (Assadi-Rad et al., 1995; Lehmann et al., 2003; García-Bocanegra et al., 2010a, 2010b; Du et al., 2012; de Sousa et al., 2014). Cats are implicated in the maintenance of *T. gondii* infection in pig farms through oocyst elimination and contamination of feed and/or water (Dubey and Beattie, 1988; Mateus-Pinilla et al., 1999; Du et al., 2012; Ichikawa-Seki et al., 2015). It has even been demonstrated that the presence of cats could increase more than eleven times the relative risk of contact with the parasite (García-Bocanegra et al., 2010b). Besides, oocysts can survive and remain infective in damp soil for more than 18 months (Du et al., 2012; Ortega-Pacheco et al., 2013). In this sense, the seroprevalence of *T. gondii* decreased significantly in pig farms where cats were vaccinated with a modified live vaccine to reduce oocyst shedding by cats (Mateus-Pinilla et al., 1999; Innes et al.,

2009; Verma and Khanna., 2013). In order to decrease the risk of *T. gondii* infection in animals, Tenter et al. (2000) advised to avoid feed and water contamination due to contact with cats. Presence of dogs has also resulted in a significant risk factor. Seropositivity in dogs is often related to the presence of cats in the farms (Arunvipas et al., 2013). The dog is an intermediate host of the parasite, and can serve as a mechanical mean of transport to *T. gondii* (Sharma et al., 2015; Gebremedhin et al., 2015).

Pig farms of this study were all under intensive management, which involves the production of pigs for sale, raised on limited space, usually with confined animal feeding operations. In intensive pig farms, the prevalence is noticeably lower in many countries (Dubey, 2009; Muraro et al., 2010; Piassa et al., 2010; Fernandes et al., 2012) as a result of the safer animal husbandry systems used on commercial farms. However, a higher risk of infection of *T. gondii* was observed in small farms (<1,000 pigs). Swine from small farms are at a greater risk for infection because they are more exposed to the infective forms of the parasite present in soil, water and various feeds (Bezerra et al., 2009). On the other hand, in the intensive farming systems, due to a higher concentration of animals raised in confinement spaces, when a failure occurs and a contamination source is present, the number of animals exposed is higher, leading to an increase in seroprevalence (Hill and Dubey, 2002; Van der Giessen et al., 2007; Lopes et al., 2013). Herd size is often correlated to management and previous studies have shown an association of herd size and seroprevalence to *T. gondii* both in pigs (Villari et al., 2009) and other livestock species (Klun et al., 2006; Vesco et al., 2007; Gilot-Fromont et al., 2009), although García-Bocanegra (2010b) did not observed this fact. Farms with larger population, good hygiene practices, intensive management and infrastructure can lead to a reduction of *T. gondii* prevalence (Villari et al., 2009;

370 Ortega-Pacheco et al., 2013). In relation to cleaning conditions, higher exposure to *T.*
371 *gondii* was related to bad farm conservation. Also, Veronesi et al. (2011) indicated the
372 importance of cleaning the facilities, and stated that the prevalence would be reduced if
373 a mechanical and chemical cleaning method were used simultaneously.
374
375 In the present study, seroprevalence of *T. gondii* was significantly lower in farms that
376 used rodent baits. Rodents are reservoirs of *T. gondii* (Dubey et al., 1995c; Weigel et al.,
377 1995; Hejlícek et al., 1997; Hill et al., 2005) and have been suggested to play an
378 important role for direct transmission in pig farms due to consumption of infected
379 rodents by pigs (Weigel et al., 1995; Kijlstra et al., 2008; Villari et al., 2009). The use of
380 baits and restriction of cats as a strategy for rodent control was significantly associated
381 with a reduced number of *Toxoplasma* seropositive pigs (Wang et al., 2002; Villari et
382 al., 2009; García-Bocanegra et al., 2010b; Hill et al., 2010). Therefore, in order to
383 reduce the risk of *T. gondii* infection in pig farms, appropriate rodent control programs
384 will have to be carried out (Hill et al., 2005; Villari et al., 2009; García-Bocanegra et al.,
385 2010b).
386
387 A monitoring and surveillance program would be reasonable to find high-risk farms and
388 implement appropriate management procedures to minimize the infection pressure. The
389 implementation of specific management procedures to reduce the risk of infection of
390 pigs can help to prevent the transmission of the pathogen to humans through pork
391 consumption.
392
393 Pork products are considered to be an important source of *T. gondii* infection in humans
394 (Tenter et al., 2000; Dubey et al., 2002; Dubey, 2009). In the present study, *T. gondii*

was detected in 73.7% and isolated from 42.1% of seropositive pigs. All pigs in which the parasite is detected in tissues are seropositive and the level of isolation increased as the antibody titer in the pig did.

Although several serological studies have been performed in pigs in Spain (García-Bocanegra et al., 2010a, 2010b), none of them have related this data to the isolation of *T. gondii* in meat. On the other hand, a previous study performed by our research group on commercial pork meat in Spain revealed an isolation rate of 8%, but the seropositivity of pigs was unknown (Bayarri et al., 2012).

The isolation rate obtained in the present study was relatively high. Viable *T. gondii* organisms were isolated from tissues of pigs collected in other countries (Dubey, 2009; Guo et al., 2015). Some authors have found a prevalence of *T. gondii* in Europe in fresh meat that varies from 0.4% in Austria (Edelhofer, 1994) to 38% in UK (Aspinall et al., 2002). However, our rate is lower than the 51.5–98% isolation rate from pigs reported in the USA (Dubey et al., 1995b, 2012). In other studies conducted in South America, the isolation rates ranged from 12.8 to 55.0% (Omata et al., 1994; Bezerra et al., 2012). Anyway, parasitological surveys based on abattoir samples carried out in America do not provide a true assessment of risk to humans, because post slaughter treatment of meat (storage and other post-harvest treatments with salt) can affect the viability of tissue cysts (Hill et al., 2004).

The high rates of positivity observed in this study compared to those reported in previous studies may be due to the fact that we have used only seropositive animals. Selection of tissues by screening donor pigs for *T. gondii* antibodies before bioassay

increased the efficiency of isolation *versus* bioassays of all tissues irrespective of antibody status of the donor pig (Dubey, 2009). Our results were similar to other studies on tissues from seropositive animals where the observed isolation rate of *T. gondii* from naturally infected pigs was 36.8% in USA (Dubey and Jones, 2008), 40.5% in Portugal (de Sousa et al., 2006) and 47.2% in Brazil (Cademartori et al., 2014), and higher than the 25% reported in Brazil by dos Santos et al. (2005), among others.

In the cases where the parasite has been detected by mouse seroconversion, it not always has been shown to be viable. These results can be due to the fact that the tissue samples of infected pigs used for this assay contained non-viable parasites (Dubey et al 1995a). Additionally, the tissue digestion step of the bioassay may reduce *T. gondii* viability (Dubey et al., 1995b, 2010). On the other hand, *T. gondii* can most likely be demonstrated in the brain (Dubey, 2009; Burrells et al., 2015). However, in some strains (e.g. the GT-1 strain), *T. gondii* persists in the lung for several weeks and can be evidenced there more easily than in the brain (Dubey, 2010). A bioassay is positive if at least one cyst is detected in the brain of any of the inoculated mice (Garcia et al., 2006; Klun et al., 2011); we performed PCR and the results showed a low parasite burden. In addition, bioassay results (titers in mice were low) suggested that pork tissues contain low levels of infective organisms. However, considering that failure to demonstrate *T. gondii* in mice does not prove lack of infection, antibodies to *T. gondii* should be sought in the sera of inoculated mice (Dubey 2010). In any case, these data underscore the need for consumer education and further measures to prevent the consumption of these meats without pre-treatment (e.g., cooking, freezing or curing) to kill the parasite.

444 A main objective of this investigation was to compare antibody test results with
445 isolation of viable *T. gondii* in tissues. Seropositivity in general is a good indicator of
446 the presence and viability of the parasite in tissues (Dubey et al., 1995b, 2002; Dubey
447 and Jones, 2008) and some authors mention that the level of isolation increases with
448 antibody titer in the pig (Dubey et al., 1995b). However, sometimes the parasite was not
449 isolated despite seropositive animals were used (Gajadhar et al., 1998). However, the
450 antibody titer that should be considered indicative of latent infection in pigs is not
451 always certain because viable *T. gondii* has been isolated from seronegative pigs
452 (Hejlícek and Litérak, 1994; Omata et al., 1994; Dubey et al., 1995b, 2002; de Sousa et
453 al., 2006). In this regard, Hill et al. (2005) suggest that the antibody response may be
454 independent of parasite burden and in some studies, no statistical association was found
455 between the titers of the tested animals and isolation in mice (dos Santos et al., 2005). In
456 our study, we found that there was a significant dependence between the titers of pigs
457 and presence and viability of *T. gondii* in the tissues. As far as we know, this research is
458 the first study carried out in Spain in which seroprevalence and isolation of *T. gondii* in
459 tissues are correlated, using bioassay to assess the risk of infection. Due to the increased
460 probability of detecting viable forms of *T. gondii* in tissues of pigs with titers $\geq 1:80$,
461 this could be the cutoff for discriminating those higher risk animals. The detection of *T.*
462 *gondii* is not possible by currently practiced meat inspection, but serological tests can be
463 used to detect *T. gondii* antibodies in pig herds and can consequently be helpful to
464 identify potentially contaminated pork, and could be used as an effective control tool for
465 the industry of cured meat products.
466
467 **Conclusions**
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Although pig titers determined in this study were very low, the widespread seroprevalence found and the subsequent assessment of risk factors confirms the importance of controlling environmental factors in order to avoid the transmission of the parasite in pig farms. Consequently, it is very important to maintain intensive management production, with minimal contact with the outside, restricting cat access inside farms, improving hygiene facilities and implementing an efficient rodent control system. This should result in farms with a very low prevalence or even in "*T. gondii* Free Farms" that will provide safer raw material for the elaboration of cured ham.

Current meat inspection at slaughterhouse cannot detect the presence of *T. gondii*. Nevertheless, due to the increased probability of detecting viable forms of *T. gondii* in tissues of pigs with titers ≥1:80, this serological titer could be the cutoff for discriminating those higher risk animals, and could be used as an effective control tool for the industry of cured meat products. In practical terms, we propose that this value could be used as a critical limit in the HACCP system.

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Table

Table 1. Risk factors associated to *T. gondii* seroprevalence in the studied farms.

Risk Factor	Variables	% IFA positive	P value
Sex of the animals	Male	28.1	>0.05
	Female	24.6	>0.05
Number of animals	<1,000	27.9	**0.010**
	1,000-2,000	21.7	>0.05
	>2,000	19.1	>0.05
Production system	Closed	20.0	>0.05
	All in/All out	25.1	>0.05
Cannibalism	No	25.0	>0.05
	Yes	27.5	>0.05
Outdoor facilities	No	22.4	>0.05
	Yes	18.8	>0.05
Conservation of farms	Good	26.4	>0.05
	Regular	21.4	>0.05
	Bad	36.7	**0.017**
Maintenance of farms	Good	23.4	>0.05
	Regular	20.0	>0.05
	Bad	32.1	**0.019**
Use of exclusive clothing	No	27.3	>0.05
	Yes	24.0	>0.05
Weeds around the farm	No	20.5	>0.05
	Yes	26.0	>0.05
Window status	Good	25.0	>0.05
	Bad	25.1	>0.05
Temperature maintenance system	No	26.1	>0.05
	Yes	24.2	>0.05
Animals inside the farm	Cats	29.2	**0.001**
	Mice	25.2	>0.05
	Birds	23.3	>0.05
Animals out of the farm	Dogs	33.7	**0.003**
	Cats	28.0	**0.001**
	Mice	26.1	>0.05
	Birds	15.0	>0.05
Rodent control	Professional	28.3	>0.05
	Baits	23.4	**0.009**
	Cats	28.1	>0.05
Feed administration	Wet	24.8	>0.05
	Dry	25.3	>0.05
Water source	Well	24.4	>0.05
	River	25.9	>0.05
	Irrigation ditch	24.3	>0.05

Table

Table 2. Pig serological titers and results of mice bioassays in tissues.

	Pig titer	*T. gondii* in pork tissues	
		Presence [a]	Viability[b]
Negative control			
	<1:20	0/16	0/0
	<1:20	0/16	0/0
	<1:20	0/16	0/0
Seropositive pigs			
	1:20	0/10 (6)*	0/0
	1:20	0/16	0/0
	1:20	3/16	0/0
	1:20	1/16	0/1
	1:20	0/16	0/0
	1:40	0/15 (1)*	0/0
	1:40	0/16	0/0
	1:40	0/16	0/0
	1:40	0/14 (2)*	0/0
	1:40	0/16	0/0
	1:40	0/14 (2)*	0/0
	1:40	1/16	0/1
	1:40	1/16	0/1
	1:40	4/14 (2)*	2/4
	1:80	3/16	0/3
	1:80	11/15	3/11
	1:80	13/15	7/13
	1.80	12/16	1/12
	1:80	11/15	2/11
	1:80	11/16	3/11
	1.80	12/16	4/12
	1:80	13/16	2/13
	1:80	10/15 (1)*	3/10
	1:80	2/10 (6)*	0/2
	1:80	0/16	0/0
	1:80	1/15 (1)*	0/1
	1:80	4/16	0/4
	1:80	2/13 (3)*	0/2
	1:80	0/16	0/0
	1:80	6/16	4/6
	1:160	10/15 (1)*	5/10
	1:160	8/16	2/8
	1.160	5/14 (2)*	0/5
	1:160	12/15 (1)*	3/12
	1:160	11/15 (1)*	3/11
	1:160	9/16	1/16
	1:160	12/16	0/12
	1:160	3/16	1/3

[a] Seropositive mice by IFA/Total of mice.

[b] Positive mice brain by PCR/Total of seropositive mice by IFA.

*Number of dead mice during the bioassay.

Figure

Figure 1. Map of Aragón (NE Spain) showing the location of the studied farms.

Figure

Figure 2. Titers obtained in the serological analysis (IFA) of 1,200 fattening pigs.