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Occurrence of *Toxoplasma gondii* in Iberian pork and its association with pig seropositivity

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ABSTRACT

Pork is recognized as a major source of *Toxoplasma gondii* infection in humans. Although the potential association between seropositivity in white pigs and the presence of *T. gondii* in their meat has been investigated, corresponding information on the Iberian pig breed is still limited. In this study, we investigated the presence of *T. gondii* in Iberian pork and assessed its correlation with individual serological profiles to evaluate whether antibody titres can serve as indicators of meat contamination. We tested the sera of 238 Iberian pigs from three southwestern Spanish provinces (Badajoz, Cáceres, and Córdoba) using an indirect immunofluorescence assay (IFA), and analyzed matched diaphragm samples by quantitative PCR (qPCR) for *T. gondii* DNA detection. Serological analysis revealed an overall seropositivity rate of 46.22 %, with significant regional differences ($p \leq 0.050$). Córdoba exhibited the highest seropositivity (57.89 %), followed by Cáceres (48.38 %) and Badajoz (35.71 %). Concurrently, *T. gondii* DNA was present in 14.29 % of the diaphragm samples, with parasite loads ranging from 78.56 to 219.09 parasites/g. A statistically significant correlation ($p \leq 0.001$) was observed between grouped IFA titres (<1:20, 1:20–1:40, ≥1:80) and qPCR positivity in the corresponding diaphragm samples. Notably, the proportion of animals with titres ≥1:80 closely matched the rate of qPCR-positive meat samples. We concluded that this serological threshold can serve as an effective screening tool to discriminate animals that are at a higher risk of harboring the parasite, thereby improving food safety within the HACCP-based safety system at the slaughterhouse and in the meat industry.

1. Introduction

Toxoplasma gondii is a zoonotic, apicomplexan parasite with a wide host range comprising virtually all warm-blooded animals. Its life cycle is heteroxenous, requiring felines as definitive hosts where sexual reproduction occurs, and a broad array of intermediate hosts, including livestock and humans, in which asexual multiplication and tissue cyst formation take place. Infections in humans are frequently asymptomatic. However, in immunocompromised individuals and during primary maternal infection in pregnancy,

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toxoplasmosis can result in severe disease, potentially leading to congenital toxoplasmosis, which, in turn, can lead to miscarriage or provoke serious health problems in the fetus (Almeria and Dubey, 2021; Attias et al., 2020; Cerutti et al., 2020; EFSA et al., 2018).

One of the major pathways of human exposure to *T. gondii* is foodborne transmission, particularly through the consumption of infected raw or undercooked meat. Among meats, pork has been identified as the most frequent source of *T. gondii* infection, followed by lamb and game, due to the relatively high prevalence of the parasite in those animals (EFSA et al., 2018; Opsteegh et al., 2016). Seroepidemiological studies have revealed a broad prevalence of *T. gondii* among pig populations, with reported seropositivity rates varying widely, from less than 1 % to over 50 %, depending on geographic region, farming practices, and detection methods (Pablos-Tanarro et al., 2018; Swanenburg et al., 2019).

Serology can be used to estimate the risk of human infection, as a clear correlation has been demonstrated between the seroprevalence of *T. gondii* in livestock specimens and the presence of tissue cysts in meat. This correlation was studied for the principal livestock species. The probability of detecting *T. gondii* parasites in seropositive animals was highest in pigs (58.8–95.8 %), followed by poultry, (53.4 %), sheep, and goats (39.4 % and 35.0 %, respectively); it was lowest in horses (8.8–13.8 %) and cattle (3.6 %) (Herrero et al., 2016; Opsteegh et al., 2016). Nevertheless, seroprevalence in pigs does not always align with the presence of tissue cysts in pork (De Berardinis et al., 2017). In consequence, further studies are still needed to substantiate a clear, robust relationship between *T. gondii* seropositivity in pigs and the presence of the parasite in pork and its derived products.

The Iberian pig (*Sus scrofa domesticus*) is a unique porcine breed with genetic characteristics that distinguish it from other members of the domestic pig species. Iberian pigs are traditionally reared in an extensive fattening system, associated with the "Dehesa" ecosystem of the southwestern Iberian peninsula. This breed is highly appreciated in the gastronomic sector; it is primarily used for meat production as well as high-quality processed products, including *jamón* (dry-cured ham), *paleta* (shoulder ham), *lomo* (cured loin), a variety of sausages, and further derivatives, including lard, pâté, and *sobrasada* (Herrero-Medrano et al., 2013). In this context, the wide variety and high quality of products derived from the Iberian pig breed have led to an elevated level of production, as well as widespread consumption in Spain and internationally. Although several studies have addressed the potential correlation between seropositivity and the presence of *T. gondii* in white pigs, data on the Iberian pig remain scarce. To the best of our knowledge, few investigations have specifically examined the detection of this parasite in Iberian pork and its correlation with serological results from individual pigs.

For this reason, our study aimed to investigate the presence of *T. gondii* tissue cysts in Iberian pork and to assess the correlation thereof with individual pig seroprevalence in order to evaluate whether serological titres can serve as potential indicators of *T. gondii* contamination in meat.

2. Materials and methods

2.1. Sampling and sample collection

Sampling was designed and carried out by the Veterinary Health Surveillance Centre of the Complutense University of Madrid (VISAVET) and a research group of the Agrarian Technological Institute of Castilla y León (ITACyL), partners of our research team in a coordinated research project that includes the current study.

A total of 238 Iberian pigs reared in extensive production systems were tested at slaughterhouses in the southwestern Spanish provinces of Badajoz ($n = 112$), Cáceres ($n = 31$), and Córdoba ($n = 95$). The pigs were slaughtered in accordance with the Spanish quality standard for Iberian meat, ham, shoulder, and loin (Real Decreto 4/, 2014), which establishes a minimum slaughter age of 14 months, an average weight of 92–115 kg at the beginning of the montanera period, and a minimum weight gain of at least 46 kg during this period exceeding 60 days. Blood and diaphragm samples were collected from each pig. Samples were individually identified to ensure traceability throughout the study. Diaphragm tissue was selected for qPCR analysis because it is considered a representative edible muscle and offers recovery rates comparable to heart and brain tissues (Kuruca et al., 2017; Gisbert Algaba et al., 2018).

Three milliliters (3 mL) of blood were collected by puncture of the jugular vein into sterile 5 mL tubes (BD Vacutainer, no additive, BD, Franklin Lakes, NJ, USA). Sera were subsequently obtained in the laboratory by centrifugation at 3500 rpm for 10 min (Hettich Universal, Germany), transferred to 1.5 mL Eppendorf tubes, and stored frozen at −20 °C until serological analysis. Diaphragm samples were collected (10–25 g) and immediately frozen until qPCR analysis.

2.2. Indirect immunofluorescence assay (IFA) of serum samples

To evaluate the seroprevalence of *T. gondii* in individual pigs, we analyzed serum samples with the MegaFLUO® TOXOPLASMA gondii kit (Eurovet Animal Health B.V., Bladel, The Netherlands).

First, serum samples were diluted at a ratio of 1:20 using a fluorescent antibody conjugate diluting buffer (VMRD, Pullman, Washington, USA) composed of phosphate-buffered saline (PBS) with 1 % bovine serum albumin (BSA) and 0.09 % sodium azide. From this starting point, serial 1:2 dilutions were prepared in the same buffer up to a final dilution of 1:1280.

Next, a volume of 10 µL from each dilution was added to the wells of the kit slides, which were then incubated in a humid chamber for 30 min in the dark at 37 °C. After incubation, they were washed twice with PBS at pH 9.0 (VMRD) by submerging them in a Coplin jar with a magnetic stirrer (Agimatic-N-Selecta) for 10 min. A specific anti-pig IgG (DakoCytomation, Glostrup, Denmark) was diluted 1:40 in PBS at pH 7.2 (bioMérieux SA, Marcy-l'Étoile, France) containing 0.2 % Evans blue (bioMérieux), and 10 µL of this solution was added to each well. The slides were then incubated again in a humid chamber, in the dark, for 30 min at 37 °C. After this incubation, the slides underwent another washing cycle as described above, this time with the jar covered to protect the fluorochrome-conjugated

anti-IgG from light exposure. A final wash with distilled water was performed for 10 min under the same conditions.

Subsequently, drops of FA mounting fluid (VMRD) were applied to the slides, which we covered with 24 × 50 mm coverslips (Deltalab). The slides were observed under a fluorescence microscope (NIKON Eclipse 80i) using a 40× objective and ultraviolet light to detect green fluorescence. The positive cut-off point for the assay was established at a serum dilution of 1:20. Positive and negative controls, from previous studies in our laboratory, were included in all tests to validate each assay.

2.3. Real-time quantitative polymerase chain reaction (qPCR) of diaphragm samples

Before performing qPCR, all diaphragm samples were digested to extract the tissue cysts of *T. gondii* that were present, followed by cell lysis and DNA extraction.

2.3.1. Sample preparation (pepsin digestion)

Pepsin digestion of diaphragm tissue was performed following the protocol described by Dubey (1998) with modifications by Bayarri et al. (2010), adapted to analyze a 10 g sample.

For each sample, 10.0 g of tissue was weighed into a stomacher bag, and 50 mL of 0.85 % saline solution (PanReac AppliChem, Castellar del Vallès, Spain) was added. The mixture was homogenized for 2 min at 260 rpm using a stomacher and subsequently transferred to a 250 mL Erlenmeyer flask. Next, 50 mL of warmed pepsin solution (37 °C, pH ≈ 1) was added (1.3 g of pepsin, 1:10,000 activity [PanReac]; 2.5 g of sodium chloride [PanReac]; 3.4 mL of 37 % hydrochloric acid; distilled water to a final volume of 250 mL). The mixture was incubated for 1 h at 37 °C in a shaking water bath (Agimatic-N, Selecta Barcelona, Spain). After incubation, the mixture was homogenized again, using a stomacher and filtered through a 180 μm pore-size sieve (CISA, Barcelona, Spain). The filtrate was collected and then centrifuged at 2800 rpm for 10 min (Centrifuge Consul 21, Orto Alresa, Madrid, Spain). The supernatant was discarded, and the sediment was resuspended in 4 mL of phosphate-buffered saline at pH 7.2 (PanReac). Subsequently, 3 mL of freshly prepared 1.2 % sodium bicarbonate solution at pH 8.3 (Sigma-Aldrich, St. Louis, MO, USA) was added, and the mixture was centrifuged again at 2800 rpm for 10 min. Finally, the supernatant was discarded, and the sediment was suspended in 1 mL of distilled water.

2.3.2. DNA extraction

For DNA extraction, we used the Maxwell® 16 Tissue DNA Purification kit and the automated Maxwell® 16 instrument (Promega, Madison, WI, USA).

First, 200 μL of the digested sample were mixed with 300 μL of lysis buffer and 35 μL of proteinase K, then transferred to dry bead tubes (MO Bio Laboratories, Carlsbad, CA, USA). This combination of physical and chemical lysis enhances DNA extraction efficiency. The tubes were then incubated with agitation at 400 rpm for 20 h; the first hour at 56 °C and the remaining 19 h at room temperature. After incubation, cartridges (included in the kit) were placed on the platform of a Maxwell® 16 instrument according to the number of samples requiring DNA extraction. Plungers were then placed on the platform, along with 200 μL of the sample and 100 μL of elution buffer. Finally, the prepared platform was introduced into the Promega Maxwell® 16 instrument for 40 min to extract DNA of *T. gondii* from the diaphragm samples.

2.3.3. qPCR procedure

For the amplification of target DNA using qPCR, we prepared a reaction mixture for each well of the plate, consisting of 10 μL of GoTaq® qPCR Master Mix (Promega), which contains Hot-Start Taq DNA polymerase and SYBR Green, 0.5 μM of each primer (forward: RocFw and reverse: RocRv) (Gracia et al., 2020), and nuclease-free water added to reach a final volume of 17.75 μL. The sequences of the primers we used are listed in Table 1. Next, 2.25 μL of DNA from each sample under study was added to the designated wells, along with adjacent wells containing a 1:10 dilution of each sample. Additionally, in each experiment, 2.25 μL of DNA from positive control and 2.25 μL of DNA from negative control were added to each plate. The qPCR procedure was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, CA, USA) under thermal cycling conditions shown in Table 2. Finally, to validate results, we generated a dissociation curve ranging from 60 °C to 94 °C with increments of 0.5 °C.

The qPCR positivity threshold was set at a Ct value of 38. Moreover, we estimated parasite load in positive samples using a calibration curve previously established by our research group (Herrero et al., 2017). This standard curve was generated using 1:10 serial dilutions of *T. gondii* ME49 strain tachyzoites, and we calculated parasite quantification based on the curve slope (3.329).

2.4. Statistical analysis

Qualitative variables were described with absolute (n) and relative (%) frequencies, and its 95 % confidence intervals calculated with the Wilson Score method (Wilson, 1927). Quantitative variables were described with mean and standard deviation (SD). Association between two qualitative variables was checked with Pearson's Chi-square test. Normality of quantitative variables was assessed

Table 1
Primers used for *T. gondii* DNA detection by qPCR (Gracia et al., 2020).

Primers	Sequences (5′ → 3′)	Amplified region	Amplicon length (bp)
RocFw	TAGACGAGACGACGCTTTCC	Repeat element	529
RocRv	TCGCCCTCTTCTCCACTCT		

Table 2
qPCR parameters for *T. gondii* DNA detection.

Cycling step	Temperature (°C)	Holding time	Number of cycles
Enzyme activation	94	7 min	1
Denaturation	94	5 s	
Annealing	55	30 s	44
Extension	72	10 s	
Final extension	72	10 min	1
Dissociation curve	60 to 94 (Δ 0.5)	–	1

with Shapiro-Wilk test, and association between a quantitative variable and a qualitative variable was evaluated with one-way ANOVA test. Statistical analyses were carried out using IBM SPSS 30.0 for Windows, and error alpha was set at 0.050.

3. Results

3.1. Toxoplasma gondii seropositivity in Iberian pigs

Table 3 shows the results of our seropositivity analysis of the 238 serum samples collected from Iberian pigs across three provinces in southern Spain (Badajoz, Cáceres, and Córdoba), with a distribution of antibody titres ranging from <1:20 to 1:1280, and using a seropositivity cut-off point of ≥1:20 to classify samples as positive.

The IFA results revealed a seropositivity rate of 46.22 % (110/238) in Iberian pigs, with the highest rate observed in the province of Córdoba, where 57.89 % (55/95) of the animals tested positive, while Badajoz showed the lowest seropositivity rate, at 35.71 % (40/112). However, it is worth noting that the highest proportion of antibody titres was observed in the province of Cáceres, with 29.03 % (9/31) of samples showing titres ≥1:80, a substantially higher proportion than in Córdoba (16.84 %) and Badajoz (10.71 %).

Statistical analysis after grouping serological titres into negative (<1:20), low positive (1:20–1:40), and high positive (≥1:80) categories revealed a significant association ($p < 0.050$) between province of origin and *T. gondii* serological titres, indicating that seropositivity and antibody levels were significantly higher in Iberian pigs from Córdoba compared to those from Badajoz.

3.2. Detection of T. gondii DNA in Iberian pork

Regarding the presence of *T. gondii* in Iberian pork, Table 4 presents the qPCR results stratified by the province of origin of the Iberian pigs, indicating the corresponding Ct values and the estimated parasite load for positive samples. These results revealed a *T. gondii* positivity rate of 14.29 % (34/238) in Iberian pork, with the highest prevalence observed in the province of Cáceres (19.35 %), followed by Córdoba (16.84 %) and Badajoz (10.71 %). Although pork from Cáceres showed the highest number of positive samples, the estimated parasite load based on Ct values was lower than that observed in pork from Badajoz and Córdoba. While the mean estimated parasite burden in positive samples from Cáceres was 101.75 parasites/g, the average loads in Córdoba and Badajoz were higher, 148.22 and 142.47 parasites/g, respectively. Nevertheless, despite the variations observed among provinces, our statistical analysis did not reveal significant differences in *T. gondii* presence based on sample origin, either in terms of positivity rates or parasite load in positive samples ($p > 0.050$).

Table 3
Samples classified according to IFA serological titres against *T. gondii* in Iberian pigs by province (number, percentage and 95 % confidence interval).

Provinces	Serological titres*							
	<1:20	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Badajoz (n = 112)	72 64.3 % (55.1 %, 72.6 %)	17 15.2 % (9.7 %, 23.0 %)	11 9.8 % (5.6 %, 16.7 %)	0 0.0 % (0.0 %, 3.3 %)	2 1.8 % (0.5 %, 6.3 %)	3 2.7 % (0.9 %, 7.6 %)	4 3.6 % (1.4 %, 8.8 %)	3 2.7 % (0.9 %, 7.6 %)
Cáceres (n = 31)	16 51.6 % (34.8 %, 68.0 %)	3 9.7 % (3.3 %, 24.9 %)	3 9.7 % (3.3 %, 24.9 %)	1 3.2 % (0.6 %, 16.2 %)	1 3.2 % (0.6, 16.2 %)	2 6.5 % (1.8 %, 20.7 %)	3 9.7 % (3.3 %, 24.9 %)	2 6.5 % (1.8 %, 20.9 %)
Córdoba (n = 95)	40 42.1 % (32.7 %, 52.2 %)	26 27.4 % (19.4 %, 37.1 %)	13 13.7 % (8.2 %, 22.0 %)	4 4.2 % (1.6 %, 10.3 %)	4 4.2 % (1.6 %, 10.3 %)	6 6.3 % (2.9 %, 13.1 %)	2 2.1 % (0.6 %, 7.4 %)	0 0.0 % (0.0 %, 3.9 %)
Total (*n* = 238)	128 53.8 % (47.4 %, 60.0 %)	46 19.3 % (14.8 %, 24.8 %)	27 11.3 % (7.9 %, 16.0 %)	5 2.1 % (0.9 %, 4.8 %)	7 2.9 % (1.4 %, 5.9 %)	11 4.6 % (2.6 %, 8.1 %)	9 3.8 % (2.0 %, 7.0 %)	5 2.1 % (0.9 %, 4.8 %)

* 95 % confidence interval calculated with Wilson Score method.

Table 4
qPCR results and estimated *T. gondii* concentration (parasites/g) in diaphragm samples from Iberian pigs by province.

Provinces	qPCR positivity*	Ct	Average Ct ± SD	Parasites/g	Average parasites/g ± SD
		33.76		201.6	
		33.76		201.6	
		34.09		160.39	
	12	34.09		160.39	
		34.09		160.39	
	10.7 %	34.13		156.01	
		34.32		136.76	
		34.34		134.88	
		34.63		110.32	
	(6.2 %, 17.8 %)	34.72		103.65	
		34.83		96.04	
Badajoz (n = 112)		34.91	34.31 ± 0.39	90.86	142.74 ± 37.65
	6	33.95		176.73	
		34.56		115.80	
Cáceres (n = 31)	19.4 %	34.60	34.95 ± 0.96	112.64	101.75 ± 49.24
		34.84		95.38	
	(9.2 %, 36.3 %)	35.01		84.78	
		36.76		25.21	
		33.64		219.09	
	16	33.66		216.07	
		33.72		207.27	
		33.86		188.11	
		33.89		184.24	
	16.8 %	34.03		167.20	
		34.03		167.20	
Córdoba (n = 95)		34.13		156.01	
		34.23	34.28 ± 0.49	145.56	148.22 ± 47.48
		34.27		141.58	
	(10.6 %, 25.6 %)	34.58		114.21	
		34.62		111.09	
		34.83		96.04	
		34.89		92.13	
		34.97		87.16	
		35.12		78.56	
Total (n = 238)	**34** **14.3 %** **(10.4 %, 19.3 %)**	**34.41 ± 0.61**		**138.09 ± 46.50**	

* Number of positive samples and percentage (95 % confidence interval calculated with Wilson Score method).

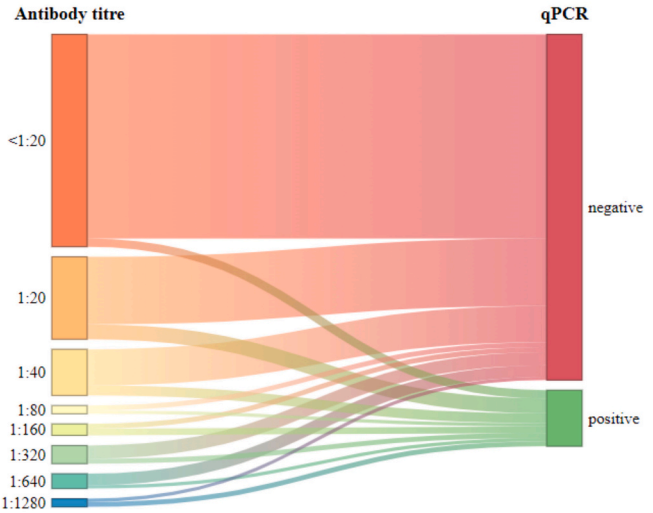

Fig. 1. Sankey diagram showing the relationship between serological titers in Iberian pigs and qPCR detection of *T. gondii* in matched diaphragm samples.

3.3. Association between seropositivity and the presence of T. gondii

After assessing *T. gondii* seropositivity in serum samples from Iberian pigs and evaluating the presence of the parasite in the corresponding pork samples, we analyzed the relationship between those two types of results to determine whether serological titres can serve as a potential indicator of *T. gondii* contamination in meat.

Fig. 1 shows the association between serological titres and qPCR results from diaphragm samples taken from the same Iberian pigs. As shown in Fig. 1, 96 % of the samples with titres below 1:20 corresponded to negative qPCR results (123/128). As antibody titres increased, the number of *T. gondii* qPCR-positive samples rose in parallel, reaching 60 % positivity among samples with a titre of 1:1280 (3/5).

We observed this positive association between grouped IFA titres (<1:20, 1:20–1:40, ≥1:80) and qPCR positivity in the corresponding diaphragm samples from the same pigs ($p < 0.001$). Pigs with low positive titres (1:20–1:80) showed a significantly higher proportion of qPCR-positive meat samples compared to seronegative animals (<1:20). This trend was even more pronounced among animals with high titres (≥1:80), as 37.5 % of them tested positive by qPCR. Notably, a significant association between serological titres and *T. gondii* qPCR positivity was consistently observed in samples and pigs from all three provinces when analyzed separately by province. When IFA titres were categorized into three levels, we observed that higher antibody titres were significantly associated with increased qPCR positivity both overall and within each province ($p < 0.050$). Notably, despite the observed association between IFA titres and qPCR positivity, statistical analysis did not reveal a significant relationship between serological titres and qPCR Ct values. This indicates that there were no significant differences in mean Ct values across the three IFA categories ($p > 0.050$). On the other hand, when the data were stratified by IFA category, no significant differences in qPCR positivity appeared among provinces within each titre group ($p > 0.050$).

4. Discussion

Our results support the assumption that toxoplasmosis remains a prevalent infection in Iberian pig farming systems. Numerous studies have investigated the seroprevalence of *T. gondii* in Iberian pigs, and results vary widely, from as low as 5.8 % at the individual level to over 50.0 % at the herd level. Despite this variability, the seropositivity observed in the present investigation (46.22 %) was notably higher than values reported in the majority of previous studies. Hernández et al. (2014) conducted a serological survey of 709 fattening Iberian pigs raised in free-range systems and reported an individual-level seroprevalence of 27.12 %, with 58.23 % of the sampled farms testing positive for *T. gondii* antibodies. Similarly, Castillo-Cuenca et al. (2020) carried out a nationwide study involving 2245 extensively raised Iberian pigs from 114 herds across Spain and reported a seroprevalence of 24.1 % using ELISA (Enzyme-Linked ImmunoSorbent Assay). Even lower prevalence values were reported by Pablos-Tanarro et al. (2018) in Iberian sows, with seropositivity rates of 5.8 % using ELISA and 8.9 % with the direct agglutination test (DAT). In contrast, our study not only revealed a higher proportion of seropositive animals, but also a substantial fraction of pigs with high antibody titres (15.54 % with titres ≥1:80).

In white pigs, typically raised in conventional intensive systems, *T. gondii* seroprevalence is usually lower than in Iberian pigs. For instance, García-Bocanegra et al. (2010) examined 1202 serum samples from conventional sows and fattening pigs in Catalonia (Spain) using DAT, reporting an individual-level seroprevalence of 19.0 %, increasing to 22.8 % in pigs older than seven weeks. Along the same lines, a comprehensive global review by Dubey et al. (2020) indicated that European conventional pig populations generally have seroprevalence levels below 20 %. However, seroprevalence rates vary widely from country to country due to differences in production models, pig breeds, husbandry conditions, and biosecurity measures. For example, a large-scale screening of over 220,000 slaughter pigs in the Netherlands between 2012 and 2016 reported consistently low seroprevalence levels, lying below 3 % (Swanenburg et al., 2019), while studies in the Czech Republic (Bártová and Sedlák, 2011) and Serbia (Klun et al., 2006), reported considerably higher rates of 36 % and 28.9 %, respectively. Such marked differences highlight the need for cautious interpretation when comparing *Toxoplasma* seroprevalence data across countries and studies.

The high prevalence of toxoplasmosis we observed in Iberian pigs may be strongly influenced by the extensive production systems in which they are typically raised. Unlike intensive systems, where eventual contact with intermediate and definitive hosts (such as rodents and cats) is more easily controlled, extensive systems are more vulnerable to environmental contamination by *T. gondii* oocysts. Several farm-level factors have been associated with increased seropositivity in Iberian pigs, including the presence of cats, number of animals, lack of rodent control, absence of bird-proofing in facilities, low infrastructure levels, on-farm feed storage, water from wells, and insufficient fencing, all of which are more common in outdoor or low-biosecurity operations (Herrero et al., 2016; Pablos-Tanarro et al., 2018). Further studies have pointed out that pigs raised in well-controlled indoor environments tend to have very low seroprevalence rates (often <1 %), whereas free-ranging or backyard pigs reared under less controlled conditions can reach seroprevalence values exceeding 60 % (De Berardinis et al., 2017; Dubey et al., 2020).

The variations observed across provinces may be influenced by multiple factors, including the type of serological test used, animal husbandry practices, and environmental conditions, such as climate and geographic location (Albuquerque et al., 2011; Bacci et al., 2015; Zhang et al., 2020). Additionally, differences in the presence and density of vector animals across production areas, along with specific biosecurity measures and management practices implemented on farms, may also contribute to the observed variability in seroprevalence (Pablos-Tanarro et al., 2018). To the best of our knowledge, no previous study has statistically compared the seroprevalence of Iberian pigs across several provinces. Therefore, this study may serve as an initial approach to investigating differences in seroprevalence in the southeastern regions of Spain, where Iberian pig farming is widespread.

We selected diaphragm tissue for qPCR analysis because that particular muscle is considered a good example of edible meat tissue (Kuruca et al., 2017; Gisbert Algaba et al., 2018): it is easy to sample, has low commercial value, and is currently the tissue used in

slaughterhouses for the detection of *Trichinella* (Commission Implementing Regulation (EU), 2015/1375), making it a practical option that is easy to integrate into routine meat inspection analysis. Results of qPCR revealed a *T. gondii* positivity rate of 14.29 % in meat samples from Iberian pigs, and it was noteworthy that the positive samples had a parasite load high enough to cause toxoplasmosis in humans, particularly in high-risk groups, such as pregnant women (Deganich et al., 2022). In this regard, qPCR detects parasite DNA without confirming whether the parasite is in its infective form. Whenever meat is not properly processed to eliminate or reduce viable parasites, this can be a potential food safety concern.

This rate of positivity aligns with findings from a comprehensive meta-analysis of *T. gondii* in meat across species, which estimated an overall pooled prevalence of approximately 12.3 % in pork by molecular detection methods (Belluco et al., 2016). Similarly, a large-scale molecular survey in Poland reported *T. gondii* DNA in tissue samples of approximately 12.2 % of seropositive pigs (Sroka et al., 2020). However, other studies have detected notably higher rates. For example, Silva et al. (2021) found *T. gondii* DNA in 66.67 % (40/60) of pig tissue samples using direct PCR, while Kuruca et al. (2017) detected parasites and/or parasite DNA in 15 out of 45 tissue samples.

Recent Spanish studies have further nuanced this picture by demonstrating that the safety of cured Iberian products and commercial dry-cured meats may vary significantly depending on production practices. Gomez-Samblas et al. (2021) reported the complete absence of *T. gondii* DNA and viable parasites in 100 % Iberian products from experimentally infected pigs after subjecting the products to a specific traditional curing process, thus showing that such protocols can effectively eliminate the parasite. Gracia et al. (2024) detected *T. gondii* DNA in 10.3 % of Spanish commercial dry-cured meat products, with the parasite viable in 6 out of 552 samples (1.1 %), suggesting that consumer risk cannot be entirely ruled out. This study included 100 samples of different Iberian dry-cured meat products, and viable Toxoplasma was detected in only one sausage sample (Iberian salchichón).

The positivity rate of *T. gondii* in Iberian pork highlights the potential risk these products may pose to immunocompromised individuals or pregnant women if the meat is not subjected to treatments capable of inactivating the parasite. Our findings substantiate the need to apply specific household measures, such as proper cooking, to inactivate *T. gondii* (EFSA et al., 2018) in fresh meat or in cases when processing methods, such as curing, do not guarantee complete parasite elimination (Herrero et al., 2017).

By comparing serological data with qPCR pork results stemming from the same Iberian pigs, we observed a positive association between the two parameters ($p < 0.050$). In other words, the likelihood of detecting *T. gondii* in meat increased in seropositive animals, particularly in those with higher antibody titres. However, the positivity rate in meat (14.29 %) was notably lower than the seropositivity observed when applying a cut-off value of 1:20 (46.22 %). Moreover, the overall proportion of qPCR-positive pork samples was similar to the seropositivity rate obtained when applying a cut-off value of 1:80 (15.54 %). These findings may support the use of a serological cut-off value of 1:80 as a potential indicator of *T. gondii* contamination in meat, since 38 % of the animals with titres ≥1:80 tested positive for the parasite in muscle tissue, whereas only 10.0 % of samples with titres <1:80 were positive-qPCR. Nonetheless, in the positive samples, we found no significant relationship ($p > 0.050$) between antibody titres and parasite load. This may indicate that a pig's immune response is not solely determined by the infectious load, but may also be influenced by intrinsic host immune factors, the specific *T. gondii* strain involved, or the time elapsed since infection. Further studies will be needed to better understand and establish a stronger correlation between antibody levels in serum and parasite burden in pork.

These findings suggest a dose-dependent relationship between serological response and tissue infection, confirming the use of antibody titres as a potential proxy for estimating the likelihood of meat contamination in Iberian pigs, regardless of geographic origin. Based on the increased probability of detecting *T. gondii* in pork from pigs with titres ≥1:80, this serological cut-off value could serve as an effective control tool for early detection and for identifying higher-risk animals. Consistent with our results, Herrero et al. (2016) observed the same relationship, reporting that 95.8 % of pigs with titres ≥1:80 harbored tissue cysts, and that the parasite was viable in 57.1 % of those cases.

Implementing this tool would allow researchers and institutions to acquire a better understanding of the epidemiological situation, as well as a more accurate assessment of the foodborne risk of *T. gondii*, with the aim of designing more effective strategies to control this hazard in the food chain. From a practical standpoint, it would be recommendable to consider a cut-off value of 1:80 as a potential critical limit within the HACCP framework.

5. Conclusions

This study provided evidence of the presence of *Toxoplasma gondii* in Iberian pork. The markedly higher probability of harboring *T. gondii* in the case of pigs with antibody titres ≥1:80 suggests that this threshold could serve as a useful indicator of meat contamination. Since current official inspection at slaughterhouse cannot detect the presence of *T. gondii*, these results underscore the viability of implementing serological screening as a practical, effective tool for identifying high-risk animals. Furthermore, the meat industry could use the proposed titre threshold of 1:80 as a critical limit within HACCP-based safety systems, thus ensuring more targeted and efficient control strategies for *T. gondii* in pork.

CRediT authorship contribution statement

Daniel Berdejo: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Paula Nieto:** Investigation, Formal analysis, Data curation. **Mª. Jesús Gracia:** Supervision, Resources, Project administration, Methodology, Conceptualization. **Ignacio de Blas:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sara Remón:** Validation, Resources. **Regina Lázaro:** Writing – review & editing, Writing – original draft, Visualization, Resources. **Susana Bayarri:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration,

Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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