Research paper

Seroepidemiology of *Toxoplasma gondii* in extensively raised Iberian pigs in Spain

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Abstract

Pigs reared under extensive farming conditions are currently in high commercial demand because they are associated with high-quality products. Nevertheless, the risk of contact with different pathogens of animal and public health concern is also higher in extensive production systems. *Toxoplasma gondii* is a widely prevalent zoonotic pathogen and transmission by contaminated pork **is likely** one of the main **routes** of human toxoplasmosis. The aim of this study was to determine the seroprevalence, risk factors and spatial distribution of *T. gondii* on extensive Iberian pig herds in Spain. Sera from 2,245 Iberian pigs **from** 114 herds were collected between 2015 and 2017 and analyzed using a commercial ELISA. The apparent individual prevalence of antibodies against *T. gondii* was 24.1% (542/2,245) and the estimated true seroprevalence was 24.3% (CI95%: 22.5-26.1). Seropositivity was detected in 86.0% (98/114; CI95%: 77.4-91.1) of 114 herds analyzed. A multi-level logistic regression model showed that *T. gondii* infection was significantly more frequent in sows **than in fattening pigs** (OR: 2.6; CI95%: 1.5-4.8) and in herds with more than three **cats compared to no cats** (OR: 2.9; CI95%: 1.1-8.7). Spatial analysis identified two statistically significant clusters of high seroprevalence. Our results indicate a widespread but heterogenous distribution of *T. gondii* in extensively reared Iberian pig herds, which may have important implications for public health through the consumption of undercooked or improperly cured pork products.

Key words: *Toxoplasma gondii*; Iberian pig; Risk factors; Seroprevalence; Spain.

1. Introduction

Toxoplasmosis is a zoonotic disease with worldwide distribution caused by *Toxoplasma gondii*, an obligate intracellular Apicomplexan protozoan capable of infecting most warm-blooded species. In humans, toxoplasmosis is usually asymptomatic, but can lead to abortions in primary infected pregnant women, congenital infection in their fetuses and severe disease in immunocompromised individuals (Dubey, 2009a). The consumption of raw or undercooked pork contaminated with *T. gondii* is a major route of transmission to humans (Guo et al., 2016; Belluco et al., 2018). Pigs can become infected by ingesting water or food contaminated with oocysts or tissues infected with cysts or congenitally.

The seroprevalence of *T. gondii* in domestic pigs varies widely from country to country and also between regions within the same country (Foroutan et al., 2019; Olsen et al., 2019), being influenced by individual factors, such as age (Villari et al., 2009; García-Bocanegra et al., 2010a; Olsen et al., 2019), management systems and/or environmental factors (Dubey, 2009b; Guo et al., 2016). Extensive production systems, in particular, have been shown to be an important risk factor for *T. gondii* exposure in pigs (Van der Giessen et al., 2007; García-Bocanegra et al., 2010b).

Spain is the second largest pork producer in the European Union (EU) and the fourth worldwide. The Iberian pig is an autochthonous breed of the Iberian Peninsula derived from the *Sus mediterraneus* with a characteristic habitat called "*dehesa*" consisting of Mediterranean holm-oak and cork-oak pastures (Garrido-Fernández and León-Camacho, 2019). Iberian pigs represent about 11% of Spanish pork production and, according to 2019 national records (MAPA, 2019), approximately 80% of the Spanish Iberian pig population is located in southwestern Spain. The Iberian pig is **raised** extensively up to the end of the fattening period (usually more than 14 months

of age) and shares its habitat and natural resources with sympatric domestic and wild species (Aparicio-Tovar and Vargas-Giraldo, 2006; Cano-Terriza et al., 2018). Meat products derived from the Iberian pig are highly appreciated and are currently in high commercial demand. Some of these products **are** consumed without cooking such as dry-cured ham, the most important meat product obtained from the Iberian pig. Viable *T. gondii* tissue cysts have been isolated from meat and ham of **pigs including dry-cured products up to 12 months of curation** (Gomez-Samblas et al., 2015; Herrero et al., 2017).

Serosurveys have been carried out in domestic pigs in Spain, with individual seroprevalence levels ranging between 16.8% and 18.8% in pigs reared indoors (García-Bocanegra et al., 2010a, 2010b; Herrero et al., 2016), and between 22.4% and 22.9% in outdoor production systems (García-Bocanegra et al., 2010a; Herrero et al., 2016). In addition, *T. gondii* exposure has also been frequently reported in humans in **this country, with seroprevalence values ranging between 12.0% and 41.4% in pregnant women (Ramos et al., 2011). Although uncommon, toxoplasmosis was also identified in transplant recipients (0.14% of 15,800) in Spanish hospitals and was the cause of mortality in 13.6% of the infected patients (Fernández-Sabé et al., 2012).** However, epidemiological information about this parasite in Iberian pig herds remains limited (Hernández et al., 2014; Pablos-Tanarro et al., 2018) and no large-scale studies have been conducted to date. Hence, the aims of the present study were: (i) to **estimate** the individual and herd seroprevalence of *T. gondii* in Iberian pigs raised under extensive management systems in Spain, (**ii**) to identify potential risk factors associated with *T. gondii* seropositivity in extensively-managed pig herds in this country.

2. Material and methods

100 *2.1. Study design*

101 From 2015 to 2017, a cross-sectional study was performed to **estimate** the
102 individual and herd prevalence of antibodies against *T. gondii* in extensively reared
103 Iberian pigs in Andalusia and Extremadura, **in** southwestern Spain (Fig. 1), the regions
104 with the largest number of Iberian pig herds population in Spain (MAPA, 2019). In
105 Andalusia, the number of herds sampled was calculated assuming an expected
106 prevalence of 50%, confidence level of 95% and absolute accuracy of 10%. A total of
107 101 herds were included in the study. Sampling was stratified by province, based on the
108 Iberian pig population for each province. Herds were selected by simple random
109 sampling from the official records of herds obtained from the Regional Government of
110 Andalusia (CAGPDS, 2019). Additionally, 13 Iberian pig herds in Extremadura were
111 sampled using a convenience sampling for logistical reasons. Whenever possible, 20
112 animals per herd, including 10 sows and 10 fattening pigs, were randomly sampled.
113 This sampling scheme allows detection of exposure with a minimum expected
114 prevalence of 15% and a confidence level of 95% (Thrusfield et al., 2018).

115 Epidemiological information was gathered by direct interview with each swine
116 farmer using a structured questionnaire. The independent variables included in this
117 study were grouped as (1) individual data: age (sows *vs.* fattening pigs) and sex; (2)
118 herd data: herd size, presence of other domestic species: cats, dogs, cattle, goats and
119 sheep; and wild species: red deer, wild boar, badgers and rodents; number of cats on the
120 farm, mortality **percentage** (at weaning, growth and breeding) and (3) biosecurity
121 measures: rodent control, disinfection, cleaning frequency and presence of perimeter
122 fences. Environmental data including mean annual temperature (ºC), mean annual
123 rainfall (mm) and mean annual humidity (%) were recorded from weather stations in the

124 **vicinity** of the sampling herds. Climatological data were obtained from the Andalusian
125 Environmental Information Network (REDIAM, 2019).
126 *2.2. Sample collection and serological analysis*
127 Blood samples were obtained by **puncture of the** *Sinus ophthalmicus*. Sera were
128 obtained by centrifugation at 400 *g* for 10 min and stored at −20 ºC until assayed.
129 Serum samples were tested by **an** indirect commercial enzyme linked immunosorbent
130 assay (ELISA) (PrioCHECK® porcine *Toxoplasma* Ab, Prionics AG, Zurich,
131 Switzerland) for **the** detection of antibodies against *T. gondii* using cell culture derived
132 **tachyzoites** as antigen. The ELISA was performed in accordance with the
133 manufacturer's instructions. Positive, weakly positive and negative control sera
134 provided by the manufacturer were included in duplicate on each plate. ELISA results
135 were expressed as percentage of positivity (*PP*), calculated according to the following
136 formula: [sample *PP* = ((sample optical density (OD) – mean negative control OD) /
137 (mean positive control OD - mean negative control OD)) X 100]. Results obtained at or
138 above the cut-off of 20 *PP* were considered positive. The sensitivity and specificity
139 values of this ELISA according to the manufacturer were 98.0% and 99.6%,
140 respectively.
141 *2.3. Statistical analysis*
142 The apparent individual seroprevalence against *T. gondii* was calculated from
143 the ratio of positive results to the total number of pigs tested. The true individual
144 seroprevalence was calculated using the Rogan-Gladen estimator (Rogan and Gladen,
145 1978) and the exact binomial confidence intervals of 95% (CI95%) were estimated by
146 the Blaker's method (Reiczigel et al., 2010).
147 The association between the independent variables and *T. gondii* seropositivity
148 was analyzed only for the region of Andalusia, where sampling was random. Variables

were first tested using a chi-square test or Fisher's exact test, as appropriate. All variables with $P < 0.20$ were selected to be included for further modeling. Cramer's V coefficients were computed pairwise to detect collinearity problems. When collinearity was detected, the variable with the clearest epidemiological relationship with *T. gondii* was retained. Finally, the effect of the selected independent variables on the response variable (seropositivity to *T. gondii*) was investigated by using a multi-level logistic regression model. The examined variables were included as fixed factors in the models with herd included as a random factor. Variables were sequentially removed if they were not significant (P-value > 0.05). The Aikake information criterion was used to assess the best model. All pairwise interactions that were biologically plausible were evaluated. The model was rerun until all remaining variables presented statistically significant values (likelihood-ratio Wald's test, $P < 0.05$). The statistical analysis was performed using the package "lme4" (Bates et al., 2015) in R software v 3.5.2 (R Core Team, 2018).

3. Results

Antibodies against *T. gondii* were detected in 542 of the 2,245 pigs tested (24.1%) with an estimated true individual seroprevalence of 24.3% (CI95%: 22.5-26.1). Seropositive animals were observed in the 86% (98/114; CI95%: 77.4-91.1) of the examined herds (Fig 1).

The distribution and univariate association of the independent variables and seropositivity to *T. gondii* are shown in Table 1. The multi-level model identified age and number of cats on the farm as risk factors associated with *T. gondii* exposure (Table 2). Individual seropositivity was higher in sows (32.9% of 678) than in fattening pigs (22.1% of 1218) ($P < 0.001$; OR: 2.6; CI95%: 1.5-4.8). Seroprevalence was also significantly higher in herds with more than three cats (42.5% of 280) compared with

174 those with no cats (22.2% of 1,078) (P = 0.048; OR: 2.5; CI95%: 1.1-8.7). **However, a**
175 **smaller number of cats (between one and three) were not found to increase the risk**
176 **of infection** (21.9% of 351) (P = 0.605; OR: 0.7; CI95%: 0.5-2.0).
177
178 **4. Discussion**
179 Pig production in free-range and organic management systems has become
180 popular and its economic importance has increased in Europe during the last few
181 decades (Früh et al., 2014). Food products derived from extensive production systems
182 such as the Iberian pig in the *dehesa* agroforestry systems are of high quality and are in
183 high commercial demand (Garrido-Fernández and León-Camacho, 2019). However, the
184 **implementation** of effective biosecurity measures and control programs for
185 transmissible diseases is difficult in these extensive systems (Davies, 2011). **This is**
186 **bound to result in** high exposure to zoonotic pathogens, including *T. gondii* **in pigs**
187 (van der Giessen et al., 2007; Kijlstra et al., 2009; Wallander et al., 2016; Cano-Terriza
188 et al., 2018).
189 The individual seroprevalence obtained in the present study showed high *T.*
190 *gondii* exposure among extensively reared Iberian pigs in Spain. Our results are
191 consistent with the 27.1% of 709 Iberian fattening pigs analyzed by ELISA in
192 Andalusia between 2008 and 2009 (Hernández et al., 2014). The high seropositivity
193 levels found in both studies in different periods suggest **an** endemic circulation of *T.*
194 *gondii* in this region. **In addition, the high herd prevalence obtained in the present**
195 **study indicates widespread *T. gondii* circulation in extensive Iberian pig farms in**
196 **Spain.** However, Pablos-Tanarro et al. (2018) found markedly lower seroprevalences
197 (ranging between 11.7% and 14.8% using ELISA and the direct agglutination test,
198 respectively) in 963 Iberian sows from five extensively managed herds in southwestern

199 Spain. Nevertheless, comparisons among studies should be made with caution given the
200 differences in number of animals or herds examined, age classes, management and
201 environmental factors and serological methods employed.

202 The overall seroprevalence found in our study was higher than the 16.6%
203 reported for pig herds reared indoors in Spain (García-Bocanegra et al., 2010b). Higher
204 seropositivity in outdoor managed pigs has been also reported in different countries
205 (van der Giessen et al., 2007; Villari et al., 2009; García-Bocanegra et al., 2010a; Limon
206 et al., 2017; Pablos-Tanarro et al., 2018). The rearing conditions of Iberian pigs, with
207 long feeding periods in the *dehesa* and limited biosecurity measures, would be expected
208 to favor contact with sporulated oocysts. In this regard, **a high seroprevalence has also**
209 **been detected in** sympatric wild boar (*Sus scrofa*) populations (between 18.6%-38.4%)
210 (Gauss et al., 2005; Calero-Bernal et al., 2016; Almería et al., 2018). *Toxoplasma gondii*
211 infections in definitive host species such as free-roaming cats (*Felis catus*), the
212 European wild cat (*Felis sylvestris*) and the Iberian lynx (*Lynx pardinus*) have also been
213 reported in the study region, suggesting the existence of sylvatic cycles for *T. gondii* in
214 Spanish Mediterranean ecosystems (Roelke et al., 2008; Millán et al., 2009; García-
215 Bocanegra et al., 2010c).

216 Individual seroprevalence was significantly higher in sows (32.9%) than in
217 fattening pigs (22.1%), which is consistent with previous reports (Villari et al., 2009;
218 García-Bocanegra et al., 2010b; Kofoed et al., 2017; Olsen et al., 2019) **and reflects the**
219 **well-known increased exposure with age and the life-long persistence of anti-*T.***
220 ***gondii* antibodies** (Dubey, 2009b). The presence of more than three cats on the farms
221 was also a risk factor for *T. gondii* seroprevalence, in agreement with the results found
222 by Meerburg et al. (2006). Domestic cats are frequently used for rodent control in pig
223 herds, having access to food, water and pig facilities. Even though the presence of cats

224 was not reported by farmers in 35 of the 114 pig herds analyzed, the entrance of free-
225 roaming cats or other wild felid species in these farms is difficult to control and
226 therefore, their presence cannot be ruled out. Cats are frequently infected and shed
227 oocysts when they are young (Dubey, 2001), and the developed immunity usually
228 prevents from re-excretion (Dubey, 1995). Consequently, **a higher** seroprevalence in
229 herds with a larger number of cats **probably reflects the higher likelihood for some of**
230 **them being juvenile**, which could be shedders and imply an additional risk of infection
231 for pigs.

232 In conclusion, our results show a high and widespread *T. gondii* exposure in
233 extensively managed Iberian pig herds in Spain. The high seroprevalence found in this
234 breed **may have important implications for public health through the consumption**
235 **of undercooked or improperly cured pork products. Although curing has been**
236 **shown to inactivate *T. gondii* cysts, its effectivity depends on salt concentration,**
237 **time and temperature (Dubey, 1997; Kijlstra and Jongert, 2008). In this regard,**
238 **the application of the minimum dry-curing times, which are defined by the**
239 **Spanish Ministry of Agriculture as 600 days for ham legs and 365 days for pork**
240 **shoulders, are likely to be effective in removing the infective capability of *T. gondii***
241 **(Gómez-Samblas et al., 2016). Ensuring an adequate dry-curing time and proper**
242 **cooking of other derived meat products can be useful measures to reduce the**
243 **zoonotic risk of *T. gondii* infections. At farm level,** management practices, such as the
244 use of rodent control methods by authorized companies instead of domestic cats, could
245 be important to reduce the risk of *T. gondii* circulation in extensively managed pig
246 herds. In addition, risk-based surveillance could also be useful to establish control
247 measures against *T. gondii* circulation in Iberian pig herds managed under extensive
248 production systems.

Conflict of interest

None of the authors of this study has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

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455 **Table 1.** Distribution of independent variables associated with *Toxoplasma gondii*
456 seropositivity in extensively **raised Iberian** pigs **(n = 1,976)** in Spain, **2015-2017.**
457 Variables with *P*-value < 0.20 in the univariate analysis were included in the multi-level
458 logistic regression models to determine potential risk factors

Variable	Category	Nº positives/overall[a] (%)	*P*-value
Age	Fattening pigs	269/1218 (22.1%)	<0.001
	Sows	223/678 (32.9%)	
Cleaning frequency	< 6 months	174/642 (27.1%)	0.511
	> 6 months	167/655 (25.5%)	
Disinfection frequency	< 6 months	186/662 (28.1%)	0.192
	> 6 months	163/654 (24.9%)	
Herd size	<127 animals	152/636 (23.9%)	<0.001
	127-280 animals	127/622 (20.4%)	
	> 280 animals	212/617 (34.4%)	
Mean annual temperature	14-16 ºC	165/802 (20.6%)	<0.001
	16-20 °C	343/1174 (29.2%)	
Mean annual rainfall	400-600 mm	150/556 (27.0%)	0.024
	601-800 mm	228/983 (23.2%)	
	801-1500 mm	150/556 (27.0%)	
Mean annual relative humidity	< 65%	238/912 (26.1%)	0.715
	> 65%	270/1064 (25.4%)	
Mortality **percentage** at breeding	< 5%	277/746 (27.1%)	<0.001
	> 5%	26/48 (54.2%)	
Mortality **percentage** at growth	< 5%	295/1024 (28.8%)	0.099
	> 5%	23/108 (21.3%)	

Mortality **percentage** at weaning	< 5%	226/808 (28.0%)	0.265
	> 5%	83/263 (31.6%)	
Number of cats on the farm	No cats	239/1078 (22.2%)	< 0.001
	< 3 cats	77/351 (21.9%)	
	> 3 cats	119/280 (42.5%)	
Presence of badgers	No	278/858 (32.4%)	<0.001
	Yes	192/951 (20.2%)	
Presence of cattle	No	171/834 (20.5%)	<0.001
	Yes	276/905 (30.5%)	
Presence of cats	No	254/1137 (22.3%)	<0.001
	Yes	214/679 (31.5%)	
Presence of dogs	No	61/425 (14.4%)	<0.001
	Yes	407/1391 (29.3%)	<0.001
Presence of goats	No	375/1598 (23.5%)	<0.001
	Yes	63/139 (45.3%)	
Presence of red deer	No	108/418 (25.8%)	<0.001
	Yes	327/1281 (25.5%)	
Presence of perimeter fences	No	7/20 (35.0%)	0.340
	Yes	455/1776 (25.6%)	
Presence of rodents	No	105/419 (25.1%)	0.651
	Yes	340/1299 (26.2%)	
Presence of sheep	No	359/1439 (24.9%)	<0.001
	Yes	89/318 (28.0%)	
Presence of wild boar	No	81/310 (26.1%)	<0.001
	Yes	354/1389 (25.5%)	

	Rodent control	No	112/469 (23.9%)	0.035
		Yes	227/773 (29.4%)	

[a] Missing values were omitted.

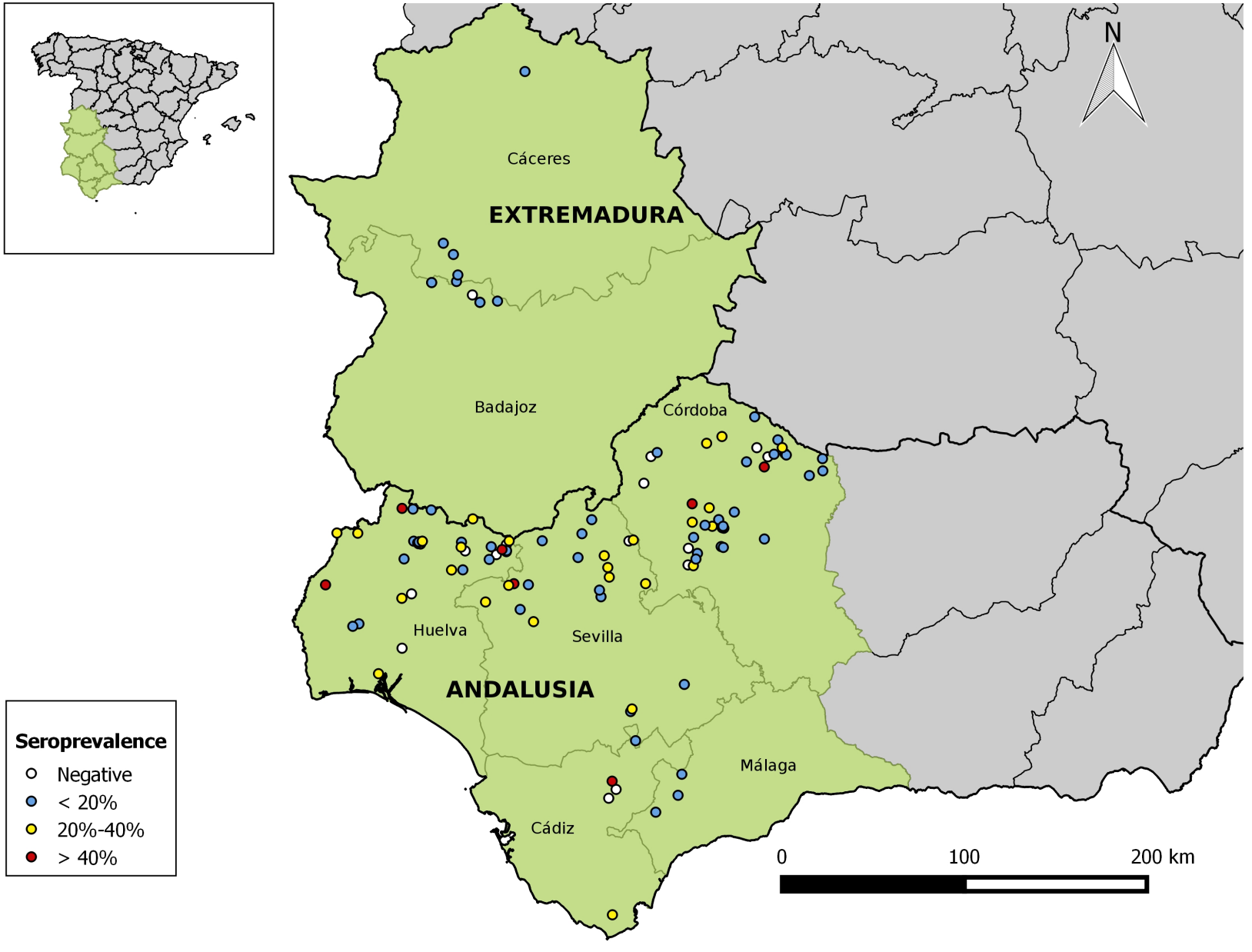
464 **Table 2**. Results of the multi-level logistic regression model of potential risk factors
465 associated with *Toxoplasma gondii* seropositivity in extensively **raised Iberian** pigs **(n**
466 **= 1,976)** in Spain, **2015-2017.**

Variable	Category	Positive/Total (%)	*β (S.E.)*	OR	CI95%	*P*-value	***P*-value[a]**
Age	Sows	223/678 (32.9)	1.0 (0.3)	2.6	1.5-4.8	<0.001	**<0.001**
	Fattening pigs	269/1218 (22.1)		**1.0**	[b]	[b]	
Number of cats on the farm	> 3 cats	119/280 (42.5)	1.1 (0.5)	2.9	1.1-8.7	0.048	**0.047**
	1-3 cats	77/351 (21.9)	-0.3 (0.5)	0.7	0.3-2.1	0.605	
	No cats	239/1078 (22.2)		**1.0**	[b]	[b]	
σ^2	**2.681**						

467 [a] Overall significance of the variable (Likelihood ratio test). [b] Reference category. **σ^2:**
468 **Variance of random effect (herd).**
469
470
471
472
473

Figure caption

Fig. 1. Map of southwestern Spain showing the distribution and within-herd seroprevalence of *Toxoplasma gondii* in the sampled pig herds.


Cáceres
EXTREMADURA
Badajoz
Córdoba
Huelva
Sevilla
ANDALUSIA
Málaga
Cádiz
N
0
100
200 km
Seroprevalence
Negative
< 20%
20%-40%
> 40%