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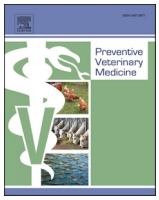

Is serology a realistic approach for monitoring red deer tuberculosis in the field?

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ABSTRACT

Tuberculosis (TB) is a zoonotic mycobacterial infection with great importance in human health, animal production, and wildlife conservation. Although an ambitious eradication programme in cattle has been implemented for decades, TB-free status has not yet been achieved in most of Spain, where animal TB persists in a multi-host system of domestic and wild hosts, including the red deer (*Cervus elaphus*). However, information on long time series and trends of TB prevalence in wildlife is scarce. The diagnosis of TB in wild red deer is often based on gross pathology and bacteriological culture confirmation, although recently serological assays have been developed to detect anti- *Mycobacterium tuberculosis* Complex (MTC) antibodies. Particularly, protein complex P22 has demonstrated to yield good specificity and sensitivity in the serological diagnosis of MTC for red deer, as well as cattle, goats, sheep, pigs, wild boar, and European badger. Thus, the objective of the present study was to compare the performance of the P22-ELISA with TB-compatible lesion detection, as well as to assess the potential application of each technique for determining spatiotemporal trends and risk factors of MTC infection in wild red deer from low and high TB prevalence areas of Spain over the last two decades. We tested 5095 sera from 13 wild populations by indirect ELISA using P22 as antigen. Mean seroprevalence (13.22%, CI_{95}: 12.32–14.18) was compared with the prevalence of macroscopic TB-compatible lesions (6.94%, CI_{95}: 6.18–7.79). The results evidenced a poor agreement between both techniques ($K < 0.3$), although generalized TB-lesions and anti-P22 antibodies showed a positive association ($\chi^2 = 9.054$, $P = 0.004$). Consequently, TB-lesion based prevalence and seroprevalence cannot be considered as equivalent for TB surveillance in red deer. Regarding the spatiotemporal trend of TB in red deer in Spain, we observed a North-South gradient of TB occurrence [North: 1.23% (CI_{95}: 0.77–1.97) of TB-lesions and 12.55% (CI_{95}: 10.91–14.41) of P22-ELISA; Centre: 7.10% (CI_{95}: 6.04–8.33) and 8.74% (CI_{95}: 7.57–10.08); South: 21.04% (CI_{95}:17.81–24.69) and 23.09% (CI_{95}: 19.73–26.84), respectively]. Overall, there was a stability over time, with higher prevalence in adults belonging to densely populated sites. We conclude that the P22-ELISA alone is not sufficiently reliable for TB surveillance in red deer at large spatiotemporal scales. Instead, we recommend combining gross pathology and P22-ELISA.

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1. Introduction

Animal tuberculosis (TB) is a zoonosis caused by bacteria of the *Mycobacterium tuberculosis* complex (MTC) (WHO, 2020). It is regarded as one of the main challenges in animal health, on account of its wide distribution, economic repercussions on livestock farming, animal movement restrictions and implications in wildlife management and conservation (Santos et al., 2012). Bovine TB persists to be endemic in most of mainland Spain, ranking third in terms of prevalence in bovine herds among European countries (EFSA, 2019). Often, TB persists thanks to the establishment of a multi-host system (Gortázar et al., 2012; Barasona et al., 2019). This is the case of the Mediterranean habitats of central-southern Iberia, where wildlife and livestock represent each almost 50% of the total host community, and MTC-infected non-bovine hosts are far more numerous than infected bovines (Santos et al., 2020). Wild boar (*Sus scrofa*), red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) are considered the main wild reservoirs in those areas, since certain populations of the referred species maintain high prevalence rates even in the absence of cattle (Vicente et al., 2006; Santos et al., 2012; Barasona et al., 2019).

The red deer has expanded both in population size and distribution throughout Europe, due to land use changes and management (Delibes-Mateos et al., 2009). Currently, the species is patchily distributed along large parts of the Iberian Peninsula (Acevedo et al., 2008). Although MTC infection has been reported in red deer worldwide (Table 1), the epidemiological relevance of the species at local scale varies according to management and habitat conditions. For instance, it has been demonstrated that at high population densities in fenced hunting estates or protected natural areas, red deer alone is able to maintain MTC circulation, even in the absence of domestic or other wild hosts (Gortázar et al., 2012, 2015; Palmer, 2013). On the other hand, information on long temporal series and trend of TB prevalence is scarce in wildlife, especially in the red deer (Vicente et al., 2013; Gortázar et al., 2015; Barroso et al., 2020).

Generally, the diagnostis of TB in wild populations of red deer is based on gross pathology as screening test and bacteriological culture as confirmation (Vicente et al., 2006, 2013; Delahay et al., 2007; Schoepf et al., 2012; Schöning et al., 2013), or exclusively based on culture (Chiari et al., 2014). Systematic inspection of target organs may include tonsils, retropharyngeal lymph nodes (LNs), lungs, pulmonary LNs, spleen, liver and mesenteric LNs (Vicente et al., 2006; Zanella et al., 2008; Martín-Hernando et al., 2010). However, *post-mortem* examination cannot always be performed in a systematic manner, so certain target regions might be overlooked. Moreover, differential diagnosis with other causative agents must be considered (Cardoso-Toset et al., 2015). In the last few decades, numerous serological assays have been developed to detect anti-MTC antibodies in wild ungulates (Chambers, 2013; Thomas et al., 2021). Among them, ELISA constitutes one of the most promising tools, since it allows to analyse numerous *ante-mortem* or *post-mortem* samples in a relatively short time and at a high cost-effectiveness (Thomas et al., 2021). Since it is a suitable diagnostic technique for large-scale screening, it constitutes a tool for TB surveillance in wild ungulate populations (Boadella et al., 2011; García-Bocanegra et al., 2012). Several MTC antigens have been tested under laboratory and field conditions aiming to achieve an accurate serological diagnosis of TB in red deer (i.e. Lyashchenko et al., 2008; García-Bocanegra et al., 2012; Thomas et al., 2019b). Recently, an immunopurified protein subcomplex derived from bovine purified protein derivative (PPD), denominated P22 (CZ Veterinaria SL, Porriño, Spain), has been shown to display fair to good specificity (Sp) and sensitivity (Se) for the detection of antibodies against MTC in cattle (Sp: 92.5 – 99.4%, Infantes-Lorenzo et al., 2019b), goats (Sp: 30.9–78%, Infantes-Lorenzo et al., 2019b), sheep (Sp: 94.4–100%, Infantes-Lorenzo et al., 2019b), pigs (Se: 84.1%, Sp: 98.4%, Thomas et al., 2019a; Sp: 100%, Infantes-Lorenzo et al., 2019b), wild boar (Se: 84.1%, Sp: 98.4%, Thomas et al., 2019a; Se: 96.7%, Sp: 100%, Fresco-Taboada et al., 2019), European badger (*Meles meles*) (Se: 76–80%, Sp: 85.7%, Infantes-Lorenzo et al., 2019a) and red deer (Se: 70.0, Sp: 99.0%, Thomas et al., 2019b). In view of the above, we hypothesized that assessment of MTC infection in red deer by means of gross pathology and by means of antibody-based tests would produce equivalent prevalence values. Thus, the objective of the present study was to compare the performance of the P22-ELISA with TB-compatible lesion detection, as well as to assess the potential application of each technique for determining spatiotemporal trends and risk factors of MTC infection in wild red deer from low and high TB prevalence areas of Spain during the last two decades.

2. Material & methods

2.1. Study areas

Sampling for the present study was carried out in thirteen sites which corresponded to hunting estates and protected areas in Northern (Asturias and Aragón), Central (Ciudad Real and Toledo) and Southern (Huelva and Sevilla) Spain. Study sites belonged to representative areas of low and high TB prevalence in cattle (MAPA, 2017) and featured a range of abundances and game management schemes in the Iberian Peninsula. The three Northern sites (N1–3) were low red deer abundance areas with no feeding and fencing, whereas one Southern (S1) and one Central (C7) site were national parks with high red deer abundance. The remaining Central (C1–6, C8) and Southern (S2) sites were fenced hunting estates with different densities and supplementary feeding levels.

2.2. Sample collection

Blood samples from 5095 hunted or captured red deer were collected between 1997 and 98 and 2019–20 hunting seasons. In hunting estates, although certain bias may be present due to hunters' selection towards adult males (Martínez et al., 2005), hunted animals are generally considered as a representative sample of the population for health monitoring of game species (Santos et al., 2012). Blood samples were obtained directly from the thoracic cavity (Boadella et al., 2011) or by puncture of the endocranial venous sinuses (Jiménez-Ruiz et al., 2016) of each animal during field necropsies or health veterinary inspections. Collected blood was kept in 10 ml sterile tubes without additives and, upon arrival to the laboratory, sera were obtained after centrifugation at 3000*g* for 10 min and stored frozen at − 20 °C until serological analyses.

2.3. Post-mortem inspection

Among the animals sampled for blood, systematic inspection of 3859 carcasses was conducted to detect TB-compatible macroscopic lesions. Typically, TB lesions or granulomas consist of an irregular to rounded lesion containing semi-solid to calcified white material (caseum), encapsulated or not, whose diameter can range from a few millimeters to several centimeters. In most sites (C2–8 and S1–2), tonsils, lungs, spleen, as well as retropharyngeal, tracheobronchial, mediastinal, and mesenteric LNs were sectioned and visually examined by professional personnel in the field (Martín-Hernando et al., 2010). Further, lesion distribution was classified in "located" when affecting only one organ and/or its draining lymph node, or "generalized" when affecting more than one organ and/or non-draining lymph nodes (European Parliament and Council, 2004). However, inspection was not complete in all sites, as not every anatomic location (i.e. head, thorax, abdomen) or every animal were inspected. For instance, in N2–3 and C1 only retropharyngeal LNs were examined, whereas in N1 not all the animals were submitted to *post-mortem* examination.

2.4. Enzyme linked immunosorbent assay (ELISA)

Sera were tested by means of an in-house indirect P22-ELISA in order

Table 1
Reported tuberculosis prevalences in deer worldwide.

Country	Region	Period	Method	Sample size	Prevalence (%)	Reference
Austria	Alpine	2009–12	Gross pathology + Bacteriological culture	590	9.32	Fink et al. (2015)
		2008–09	Gross pathology + Histopathology + Bacteriological culture + PCR	143	6.29	Schoepf et al. (2012)
France	Normandie	2001–10	Gross pathology + PCR	453	14.30	Hars et al. (2010)
		2001–02	Gross pathology + Bacteriological	72	13.00	Zanella et al. (2008)
		2005–06	culture + PCR	138	24.00	
	Bourgogne	2003–07	Gross pathology	284	0.35	Hars et al. (2010)
Germany	Alpine	2009–12	Gross pathology + Bacteriological culture	278	1.08	Fink et al. (2015)
Italy	Alpine	2009–12	Gross pathology + Bacteriological culture	514	0.19	Fink et al. (2015)
	Valcamonica	2011	Gross pathology + Bacteriological culture + PCR	53	1.89	Chiari et al. (2014)
Ireland	County Wicklow	2014–15	DAFM	?	16.00	More et al. (2017)
		ongoing		121*	8.30	
		ongoing		32*	0.00	
		2017–18	Regional Veterinary Laboratories	73*	4.10	
	County Sligo	2014–16	Gross pathology + Bacteriological culture	17*	0.00	Doyle et al. (2018)
Portugal	Idanha-a-Nova, Vila Velha de Ródão, Penamacor, Castelo Branco	2011–14	Gross pathology[a] + Bacteriological culture	3733	5.90	Madeira et al. (2017)
	Moura-Barrancos	2009–11	Bacteriological culture	163	5.52	Santos, personal communication
	Idanha-a-Nova	2011	Bacteriological culture	45	28.89	
		2008–09	Gross pathology + Histopathology + Bacteriological culture + PCR	339	11.80	Vieira-Pinto et al. (2011)
	Castelo Branco	2009–13	Bacteriological culture + PCR Histopathology	115	38.30	Matos et al. (2016)
Spain	Doñana National Park (Andalucía)	2006–2018	Gross pathology	642	42.5	Barroso et al. (2020)
		2015	Serology	101	14.3	Jiménez-Ruiz et al. (2021)
	Andalucía	2013–16	Serology	934	10.50	Cano-Terriza et al. (2018)
		2009–12	PCR	209*	2.90	Consejería de Medio Ambiente y
			Gross pathology	627*	8.50	Ordenación del Territorio (Junta de Andalucía), 2013
		2006–10	Serology	530	4.00	García-Bocanegra et al. (2012)
		1998–03	Bacteriological culture + PCR	168	21.43	Romero et al. (2008)
	Castilla-La Mancha	2013–16	Serology	427	11.60	Cano-Terriza et al. (2018)
		2005–11	Gross pathology + Bacteriological culture + PCR	365	17.75	Queirós et al. (2016)
		2000–12	Gross pathology + Bacteriological culture	2759	9.40	Vicente et al. (2013)
		1999–04	Gross pathology +Bacteriological culture	1368	13.70	Vicente et al. (2019)
	Castilla y León	2011–15	Gross pathology + Bacteriological culture	1515	2.24	Gortázar et al. (2015)
	Extremadura	2007–09	Gross pathology +Bacteriological culture + PCR	551	3.10	Castillo et al. (2011)
		1992–04	Gross pathology[a]	36,144	1.09	Hermoso de Mendoza et al. (2006)
Switzer-land	Alpine	2009–12	Gross pathology + Bacteriological culture	273	0.00	Fink et al. (2015)
	whole country	2009–11	Gross pathology + Bacteriological culture + PCR	269	0.00	Schöning et al. (2013)
UK	South-Western	?	Gross pathology + Bacteriological culture + PCR	196	1.02	Delahay et al. (2007)
Canada	Riding Mountain National Park	1997–2008	Gross pathology + Histopathology + Bacteriological culture + PCR	2347	0.38	Shury and Bergeson (2011)
				592	0.00	
		2001–08		241	11.62	
	Ontario and Alberta	1989–92	Gross pathology + Histopathology + Bacteriological culture	428°	14.95	Rohonczy et al. (1996)
New Zealand	North Island	2008–12	Gross pathology + Bacteriological culture	872	0.00	TB-free program New Zealand
	whole country	1994–97	Official Meat Inspection Scheme	80,457	0.26	
		2008–12		74,701	0.03	

PCR: polymerase chain reaction; DAFM: Department of Agriculture, Food and the Marine; *: cervids in general (mostly red deer, but also roe deer, fallow deer and sika deer where present); °: elk and red deer; [a]: Gross pathology was examined in the framework of routine government veterinarian inspections (if not signalled, it was performed by the researchers).

to detect antibodies against MTC, following the protocol described by Thomas et al. (2019a). Briefly, 96-well plates coated with the antigen (P22 at 10 μg/ml) in phosphate buffered saline solution (PBS; Panreac Química S.L.U., Barcelona, Spain) were stored overnight at 4 °C. The wells were subsequently washed with PBS solution containing 0.05% Tween-20 (PBST) (Tween 20; Sigma-Aldrich Quimica SA®, Madrid, Spain) and blocked with 5% skimmed milk powder solution in PBS (SM) for 1 h at room temperature (RT). Then, sera were added in duplicate at a dilution of 1:10 in SM, incubated stationary for 1 h at 37 °C and subsequently washed three times with PBST. Protein G horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich Química SA®, Madrid, Spain) was later added at a concentration of 0.002 mg/ml in PBS and the plates were incubated for 1 h at RT. After four washes with PBST, the color was developed with o-phenylenediamine- dihydrochloride substrate (FAST OPD; Sigma-Aldrich Química SA®, Madrid, Spain) incubated for 20 min in darkness at RT. The reaction was stopped with H_2SO_4 3 N and the optical density (OD) was measured in a spectrophotometer at 450 nm. Sample results were expressed as an ELISA percentage (E%) which was calculated by the following formula: [sample E% = (mean sample OD/2 x mean of negative control OD) x 100]. The cut-off value was calculated using a ROC analysis and established as the ratio of the mean sample OD to the double of mean OD of the negative control. Therefore, serum samples with E% values greater than 100 were considered positive (Thomas et al., 2019b). Positive and negative controls were tested in quadruplicate on every plate. Positive control sera were obtained from animals previously confirmed as TB positive based on the compatible lesions, *M. bovis* positive culture and OD> 1 at P22-ELISA; whereas negative controls were obtained from animals previously confirmed as TB-free based on the absence of compatible lesions, negative culture and with OD < 0.2 at P22-ELISA.

2.5. Statistical analysis

The agreement between P22-ELISA and TB-like lesions in each area (e.g. North, Centre, South) was calculated with Cohen's kappa coefficient (K) using Epitools program (Ausvet, Bruce, Australia). Confident Intervals with 95% confidence (CI_{95}) for seroprevalences and TB lesions-based prevalence were also calculated with Epitools. Likewise, the relation between lesion distribution (located or generalized) and antibody production (P22-ELISA negative or positive) was assessed through Pearson's chi square. On the other hand, differences in the status of TB at individual level based on P22-ELISA or TB-like lesions simultaneously (response variable: ELISA+/Lesions+, ELISA+/Lesions-, ELISA-/Lesions+, ELISA-/Lesions-) in relation to sex class, age class, and level of density and management (fixed independent factors) were analyzed by a Generalized Linear Mixed Model (GLMM) with multinomial error and logit link function. Study site and hunting season were included as random effect factors. Differences were considered statistically significant when $P \leq 0.05$. Animals from N1–3 and C1 were excluded from the analysis, as complete information regarding the presence of lesions was not available. The flow of participants used in this study is summarized in a diagram in Supplementary material (Figure S-1).

2.6. Ethical statement

The present study did not involve purposeful killing of animals, and the blood and tissue samples were not collected specifically for this study. All the animals were either legally hunted under Spanish and EU legislation with appropriate permits during the hunting season (October to February) or culled within population control programs of reserves. Protocols, amendments, and other resources were done according to the guidelines approved by each Autonomous government following the R. D.1201/2005 of the Ministry of Presidency of Spain. Sample collection was being performed by professional personnel for routine procedures before the design of the study in compliance with the Ethical Principles in Animal Research. Thus, no ethical approval was deemed necessary.

3. Results

3.1. Performance of P22-ELISA and TB-like lesions for TB diagnosis in red deer

We calculated TB prevalence in 13 wild red deer populations based on TB-like lesions and P22-ELISA, which are both imperfect in terms of Se and Sp. Prevalence based on macroscopic TB-compatible lesions mean was 6.94% (CI_{95}: 6.18–7.79) and seroprevalence mean was 13.22% (CI_{95}: 12.32–14.18). Overall, 83.57% of animals did not present TB-like lesions nor anti-P22 antibodies, whereas 2.61% of them resulted positive to both techniques. Cohen's kappa coefficient per area revealed that the agreement between the evaluated assays for TB diagnosis in red deer ranged between "low" (K < 0.20) and "fair" (0.21 < K < 0.4) (Table 2). All in all, the global kappa value was 0.21 (i.e., "fair") and it was higher when including only sites where *post-mortem* inspection was complete (n = 9; K = 0.2925; $IC_{95\%}$: 0.2350–0.3500). Further, TB-lesions distribution and antibody production showed a positive relation (Pearson's $\chi^2 = 9.054$, $P = 0.004$).

3.2. Spatiotemporal trend of TB in red deer in Spain

3.2.1. Spatial trend

Prevalence estimated by TB-like lesions and by P22-ELISA for each study site is represented in Fig. 1. The presence of TB-compatible lesions was more frequent in Southern sites (S1: 10.29%, $IC_{95\%}$: 7.22–14.48%; S2: 62.64%, $IC_{95\%}$: 56.68–68.25%), whereas it was nearly sporadic in Northern sites (N1: 0.00%, $IC_{95\%}$: 0.00–0.63%; N2: 2.05%, $IC_{95\%}$: 0.99–4.16%; N3: 2.50%, $IC_{95\%}$: 1.36–4.54%). In the centre of the country, the scenario was more heterogenous, with prevalence based on TB-like lesions ranging from below 1% (e.g. C5: 0.81%, $IC_{95\%}$: 0.22–2.9%) to above 20% (e.g. C3: 22.96%, $IC_{95\%}$: 18.24–28.47%). In most study sites (N1–2, C2–8, S1), TB seroprevalence was higher than the prevalence based on TB-like lesions, except in one Northern (N3: 1.22%, $IC_{95\%}$: 0.52 – 2.83%), one Central (C1: 1.76%, $IC_{95\%}$: 0.93 – 3.32%) and one Southern (S2: 25.35%; $IC_{95\%}$: 20.67 – 30.67%) site.

3.2.2. Temporal trend

In all three regions (North, Centre, South), the presence of TB-compatible lesions remained stable during the study period, but TB seroprevalence showed a heterogenous pattern (Fig. 2). Only a slight decrease in seroprevalence over time was observed in central Spain (R^2=*0.39, P = 0.001*). Regarding study sites, C9 displayed a significant increase in the presence of TB-lesions (R^2=*0.40, P = 0.029*) and C4 displayed a significant decrease in seroprevalence (R^2=*0.29, P = 0.033*).

3.3. Risk factors of TB prevalence in red deer

Table 3 shows the summary of the GLMM of risk factors affecting P22-ELISA positivity and TB-like lesion presence. The likelihood of resulting positive to any of the tests was higher in adults and in high-density sites. In particular, red deer from highly-populated sites

Table 2
Cohen's kappa coefficient values with 95% confidence intervals ($CI_{95\%}$) of agreement between P22-ELISA and tuberculosis-like lesions in red deer for each study area.

Study area	Sample size	Kappa value	CI_{95}
North	1378	-0.0122	-0.0333–0.0089
Centre	1944	0.2743	0.2029–0.3457
South	537	0.2479	0.1540–0.3417

Level of agreement was categorised as follows: poor agreement: K < 0.20; fair agreement: K = 0.21–0.4; moderate agreement: K = 0.41–0.6; good agreement: K = 0.61–0.8; very good agreement: K = 0.81–1.
Sample size indicates individuals with both serology and pathology.

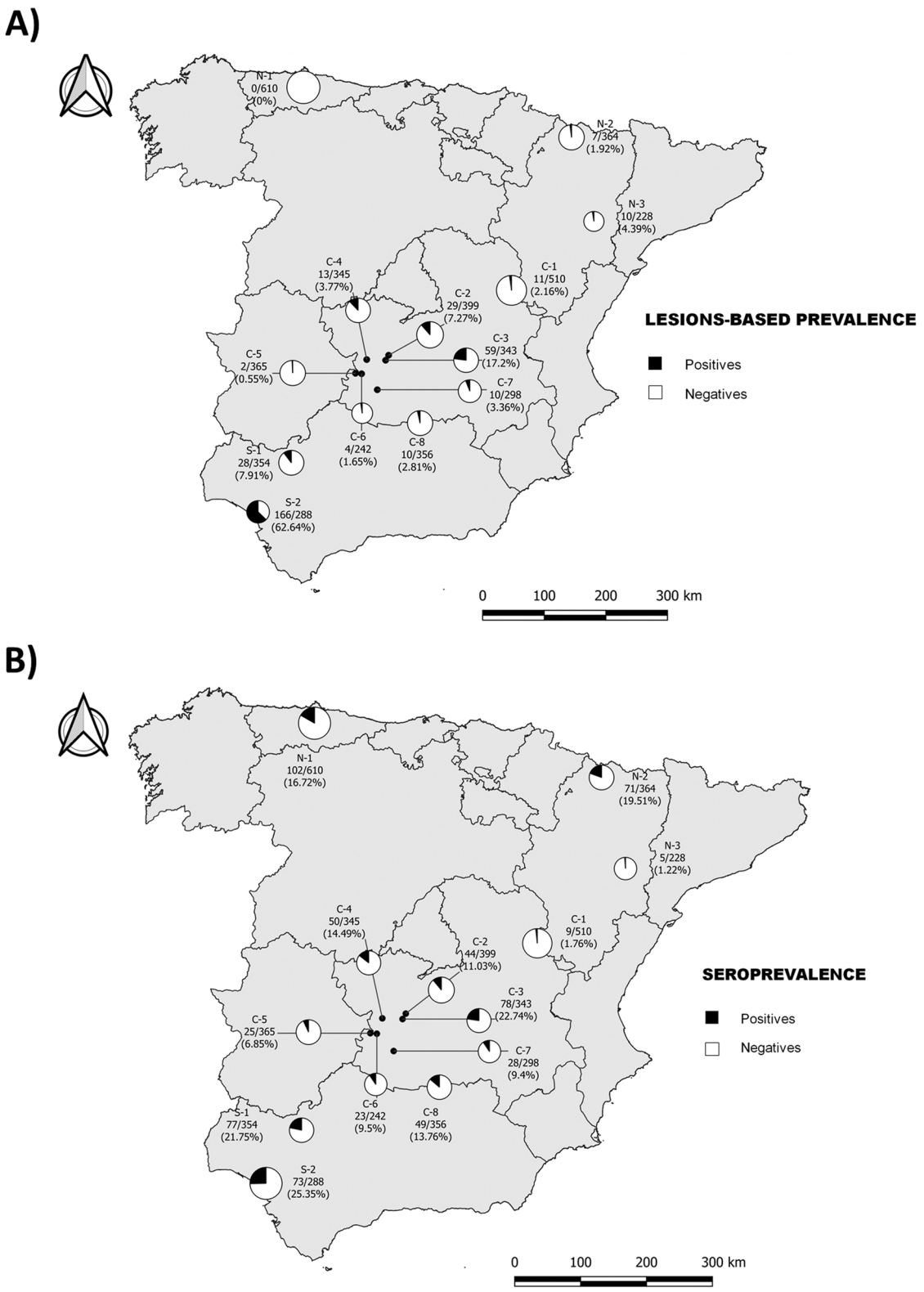

Fig. 1. Prevalence estimated by tuberculosis-like lesions (A) and P22-ELISA (B) for each study site (N1–N3, C1–C8, S1–S2) during the study period. Pie charts indicate the number of positive (black) and negative (white) red deer per site.

without management ($P = 0.030$), yearlings ($P = 0.017$) and adults ($P = 0.030$) had significantly higher risk of presenting TB-like lesions alone; whereas animals from high-density sites regardless of the management (unmanaged: $P = 0.071$; highly managed: $P = 0.021$), subadults ($P = 0.030$) and adults ($P = 0.044$) had significantly higher risk of presenting anti-P22 antibodies alone. Similarly, positivity to both P22-ELISA and TB-like lesions was influenced by age, density, and sex, being more likely in adults ($P = 0.023$) and high-density sites regardless of management ($P = 0.011$; $P = 0.027$) and less likely in females ($P = 0.016$).

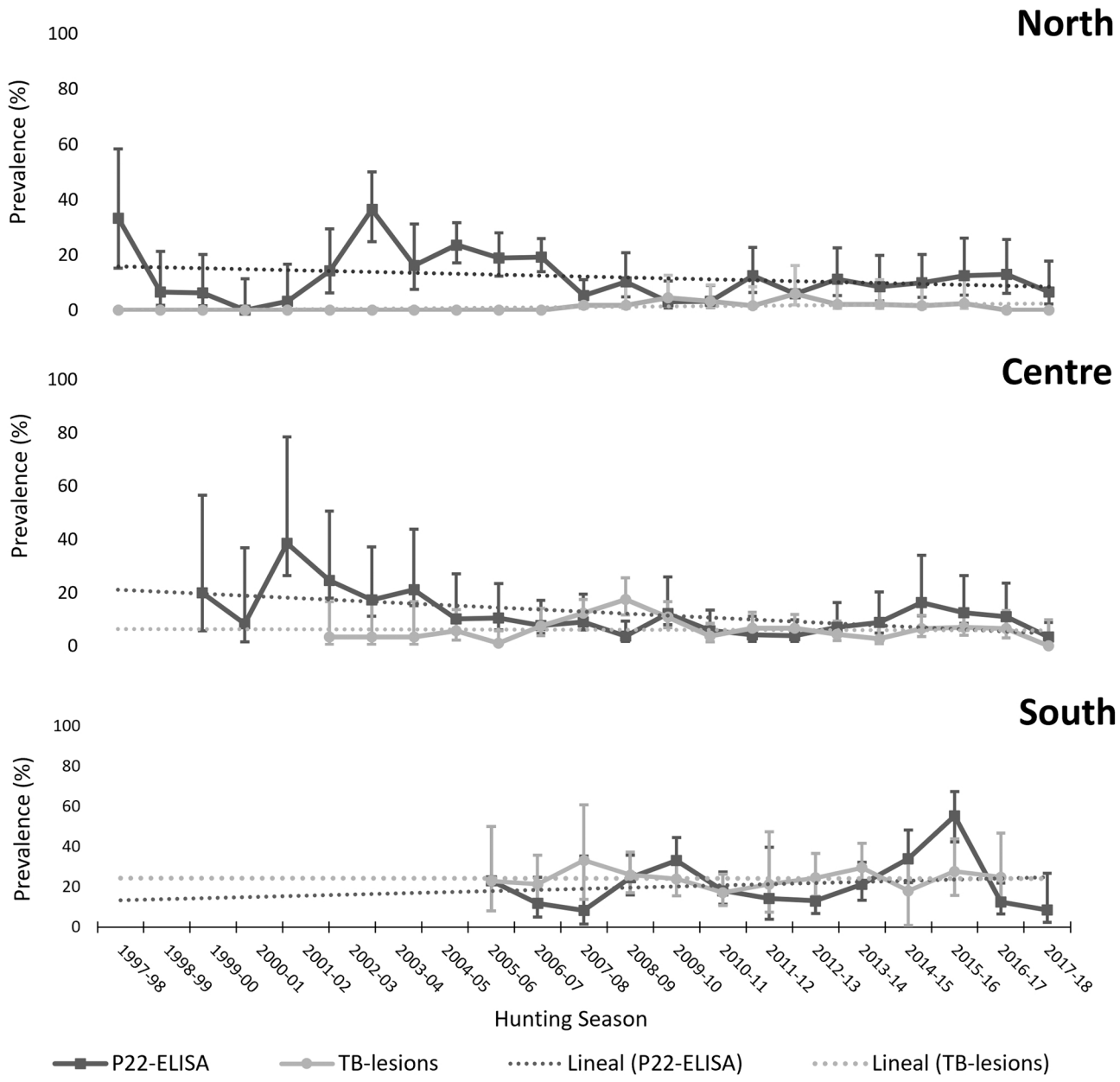

Fig. 2. Temporal trend of tuberculosis prevalence estimated by P22-ELISA and tuberculosis-lesions in each area.

Table 3
Summary of Generalized Linear Mixed Model for P22-ELISA and tuberculosis-like lesions.

	ELISA-/LES+ vs ELISA-/LES-			ELISA+/LES- vs ELISA-/LES-			ELISA+/LES+ vs ELISA-/LES-		
	Odds Ratio	CI_{95}	Pr (>|z|)	Odds Ratio	CI_{95}	Pr (>|z|)	Odds Ratio	CI_{95}	Pr (>|z|)
Intercept	0.003	0.000–0.029	0.000***	0.008	0.002–0.038	0.000***	0.002	0.000–0.024	0.000***
Females	0.684	0.454–1.030	0.069	0.844	0.578–1.231	0.378	0.557	0.346–0.897	0.016*
Yearlings	5.230	1.333–20.510	0.017*	2.101	0.786–5.616	0.139	1.188	0.182–7.735	0.857
Subadults	2.719	0.393–18.789	0.310	3.603	1.131–11.482	0.030*	2.469	0.369–16.519	0.351
Adults	7.435	2.178–25.381	0.001**	2.184	1.021–4.672	0.044*	4.033	1.209–13.461	0.023*
High density +no management	11.574	1.267–105.729	0.030*	4.826	0.875–26.615	0.071*	25.89	2.144–312.685	0.011*
Medium density low + management	0.456	0.048–4.356	0.495	4.536	0.963–21.355	0.056	1.387	0.106–18.181	0.803
High density high + management	3.665	0.481–27.896	0.210	5.953	1.310–27.050	0.021*	3.665	1.367–167.109	0.027*

Intercept: males, calves, low density & no management.
ELISA: P22-ELISA; LES: macroscopic TB-compatible lesions; +: positive; -: negative; CI_{95}: Odds Ratio 95% confidence interval

4. Discussion

The present study constitutes the most extensive spatiotemporal series of TB prevalence in wild red deer in Spain and provides additional insight regarding TB trends in Iberian wildlife. Yet, our results indicate that assessing TB prevalence in red deer by means of gross pathology and

serological assays cannot be regarded as analogous, at least for field-samples from large-scale surveys.

The selection of an appropriate diagnostic test is based on numerous factors, such as the purpose of the diagnosis and the stage of the disease, as well as diagnostic accuracy, ease of performing and economic feasibility of the test. Since antibody production and lesion development are mainly produced in advanced stages of TB infection (Pollock and Neill, 2002), it has been observed that both are closely related in other ruminants such as cattle (Waters et al., 2006, 2011) and reindeer (Waters et al., 2005), as well as in pigs (Cardoso-Toset et al., 2017) and wild boar (Aurtenetxe et al., 2008; Garrido et al., 2011; Thomas et al., 2019a). In red deer, we expected similar outcomes but, conversely, we observed only low-to-fair agreement between TB-compatible lesions and anti-MTC antibodies. Thus, in veterinary sciences, the species tested can be a determining factor for the choice of the diagnostic test. For instance, serology has been widely highlighted as a diagnostic tool of choice in both domestic pigs and wild boar (Thomas et al., 2021), since swine boast robust antibody production in response to MTC infection (Fresco-Taboada et al., 2019; Miller et al., 2019; Thomas et al., 2019a; Sridhara et al., 2021). However, cervids display weaker and later humoral responses to MTC infection (Koo et al., 2005; Waters et al., 2006). Although we observed a slight association between generalized lesions and ELISA positivity, correlation between antibody titres and lesion severity in the red deer has only been precisely demonstrated in experimental infection (Thomas et al., 2017). Besides, wild ruminants are commonly exposed to *M. avium* and other environmental mycobacteria, which may induce cross-reactivity in serological tests that detect anti-MTC antibodies, acting like a confounding factor in TB diagnosis (Buddle et al., 2010; Queiros et al., 2012; Thomas et al., 2021). On the other hand, milimetric lesions or those located in non-inspected organs may be missed during *post-mortem* examination (Gavier-Widén et al., 2009; Martín-Hernando et al., 2010), especially when it is not performed in an standardized manner by experienced personnel. Likewise, non-tuberculous mycobacteria and purulent bacteria may produce abscesses that are macroscopically indistinguishable from granulomas caused by the MTC (Muñoz-Mendoza et al., 2016; Queiros et al., 2012).

Culture, the gold standard test, remains the most reliable tool aiming to achieve an accurate TB diagnosis, due to its excellent Sp (Cousins and Florisson, 2005). Nevertheless, its application for TB monitoring in wildlife may be limited, as it is time-consuming and expensive compared to lesion assessment and serology (Thomas et al., 2021). Therefore, the combination of macorscopic TB-compatible lesions assessment with serology arises as a reasonable alternative for large scale studies.

In general, the spatiotemporal trend of TB in red deer in Spain showed a North-South gradient and a stability over time, in agreement with previous surveys based on gross pathology (Vicente et al., 2006, 2013; Gortázar et al., 2008, 2017; Barroso et al., 2020). The adults belonging to highly-populated sites were more likely to present TB-like lesions and/or MTC-antibodies, in line with risk factors associated to TB occurrence in red deer in previous local surveys based on gross pathology (Vicente et al., 2007) or serology (García-Bocanegra et al., 2012; Jiménez-Ruiz et al., 2021). All in all, and despite the apparent disagreement observed between methodologies studied, the fact that TB-lesions and P22-ELISA were influenced by the same variables suggests that both approaches might be equally valuable for applying in spatiotemporal (Vicente et al., 2013) and risk-factor assessment (Vicente et al., 2007; García-Bocanegra et al., 2012; Barroso et al., 2020) studies.

Among the limitations of this study, two points ought to be highlighted. First, post-mortem examination did not include all anatomic regions in every site. Therefore, Se may have been reduced in certain study areas (Martín-Hernando et al., 2010), particularly in northern sites. Accordingly, systematic and complete inspection is a must when aiming to implement TB monitoring based on gross pathology in the red deer (Vicente et al., 2006; Gortázar et al., 2011a, 2011b). Second, serum quality was not always optimal, especially when blood had been taken directly from the thoracic cavity (Jiménez-Ruiz et al., 2016) or when sera had been frozen and de-frozen repeatedly (Boadella and Gortázar, 2011), which may have influenced ELISA results.

5. Conclusions

Neither gross pathology nor serology arise as suitable stand-alone tools for large scale TB monitoring in red deer. When aiming to implement large scale TB monitoring programs in wild red deer, and culture is not an option due to budget or logistic constraints, a combination of macroscopic TB-compatible lessions and P22-ELISA could represent a suitable alternative. Further research on serological assays for red deer is imperative before considering serology as a reference diagnostic technique for the species under field conditions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2022.105612.

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