Occurrence and limited zoonotic potential of *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* infections in free-ranging and farmed wild ungulates in Spain

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Abstract

Little information is currently available on the occurrence and molecular diversity of the enteric protozoan parasites *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* in wild ungulates and the role of

these host species as potential sources of environmental contamination and consequent human infections. The presence of these three pathogens was investigated in eight wild ungulate species present in Spain (genera *Ammotragus*, *Capra*, *Capreolus*, *Cervus*, *Dama*, *Ovis*, *Rupicapra*, and *Sus*) by molecular methods. Faecal samples were retrospectively collected from free-ranging (n = 1,058) and farmed (n = 324) wild ungulates from the five Spanish bioregions. Overall infection rates were 3.0% (42/1,382; 95% CI: 2.1–3.9%) for *Cryptosporidium* spp., 5.4% (74/1,382; 95% CI: 4.2–6.5%) for *G. duodenalis*, and 0.7% (9/1,382; 95% CI: 0.3–1.2%) for *B. coli*. *Cryptosporidium* infection was detected in roe deer (7.5%), wild boar (7.0%) and red deer (1.5%), and *G. duodenalis* in southern chamois (12.9%), mouflon (10.0%), Iberian wild goat (9.0%), roe deer (7.5%), wild boar (5.6%), fallow deer (5.2%) and red deer (3.8%). *Balantioides coli* was only detected in wild boar (2.5%, 9/359). Sequence analyses revealed the presence of six distinct *Cryptosporidium* species: C. *ryanae* in red deer, roe deer, and wild boar; *C. parvum* in red deer and wild boar; *C. ubiquitum* in roe deer; *C. scrofarum* in wild boar; *C. canis* in roe deer; and *C. suis* in red deer. Zoonotic assemblages A and B were detected in wild boar and red deer, respectively. Ungulate-adapted assemblage E was identified in mouflon, red deer, and southern chamois. Attempts to genotype samples positive for *B. coli* failed. Sporadic infections by canine- or swine-adapted species may be indicative of potential cross-species transmission, although spurious infections cannot be ruled out. Molecular evidence gathered is consistent with parasite mild infections and limited environmental contamination with (oo)cysts. Free-ranging wild ungulate species would not presumably play a significant role as source of human infections by these pathogens. Wild ruminants do not seem to be susceptible hosts for *B. coli*.

Keywords:

Enteric protozoan parasites, Wildlife, Disease transmission, Occurrence, Molecular diversity, Genotyping, Zoonoses

Abbreviations

No keyword abbreviations are available

Data availability

The data that supports the findings of this study are available within the main body of the manuscript.

~~1.~~1 I~~NTRODUCTION~~ntroduction

Ungulates are suitable hosts for numerous zoonotic pathogens representing a public health concern (Palmer et al., 2017; Trimmel and Walzer, 2020). In Europe, wild ungulate species have increased their densities and expanded their habitat ranges during the last few decades due to land-use changes (Carpio et al., 2021). Human-driven overabundance of wild ungulate populations might increase the interaction with other animals and the risk of zoonotic transmission. Among parasitic agents, the enteric protozoa *Cryptosporidium* spp. and *Giardia duodenalis*, and, to a lesser extent, the ciliate *Balantioides coli* can infect a wide variety of ungulate species (Feng and Xiao, 2011; Ponce-Gordo and García-Rodríguez, 2021; Santin, 2020; Zahedi et al., 2015). Indeed, diarrhoea caused by both *Cryptosporidium* and *G. duodenalis* infections has been associated with significant economic losses due to growth retardation and mortality in infected livestock (Hatam-Nahavandi et al., 2019; Santin, 2020). *Balantioides coli* is the only ciliate known to infect humans. In addition, this parasite is commonly found in domestic and wild swine populations, suggesting that these animals are the main reservoir of this pathogen (Ponce-Gordo and García-Rodríguez, 2021). All three parasite species are faecal-orally transmitted through cysts (*G. duodenalis* and *B. coli*) or oocysts (*Cryptosporidium* spp.) either by direct contact with faeces of infected humans or animals or indirectly by ingestion of contaminated water or foodstuffs.

Currently, at least 46 *Cryptosporidium* species are considered taxonomically valid (Ježková et al., 2021; Ryan et al., 2021; Zahedi et al., 2021), of which ten species/genotypes have been reported in cervids: *C. parvum*, *C. bovis*, *C. ryanae*, *C. ubiquitum*, *C.* deer genotype, *C. muris*, *C. andersoni*, *C. parvum* genotype II, *C.* cervine genotype, and *C. suis*-like genotype (Hatam-Nahavandi et al., 2019). On the other hand, only *C. parvum*, *C. suis* and *C. scrofarum* have been reported in wild boar (*Sus scrofa*) to date (García-Presedo et al., 2013b; Ryan et al., 2021).

Giardia duodenalis is one of the most common enteric parasites that infect humans as well as domestic and wild mammals (Feng and Xiao, 2011). This pathogen is considered as a species complex that comprises eight (A to H) genetic variants or assemblages (Cai et al., 2021). Assemblages A and B are able to infect a wide range of hosts

including humans, livestock, companion animals, and wildlife and are considered zoonotic. Assemblages C and D are mainly identified in canids, E in domestic and wild hoofed animals, F in felids, G in rodents, and H in marine mammals (Cai et al., 2021). Besides assemblage E, assemblages A and to a lesser extent B and C have been reported in ungulate species (Cai et al., 2021).

Comparatively, the molecular diversity of *B. coli* is more limited than that observed in the *Cryptosporidium* and *Giardia* genera. To date, three (A to C) genotypes have been reported. Genotypes A and B are found in various host species, whereas genotype C seems to be restricted to non-human primates (Ponce-Gordo et al., 2011). However, little is known about the role of animal host species other than ~~of~~ non-human primates or ~~non~~ swine ~~animals~~ as suitable hosts for *B. coli*.

In Spain, data on the occurrence and molecular diversity of *G. duodenalis*, *Cryptosporidium* spp., and *B. coli* in ungulate species are limited. Most studies have reported the presence of these parasites in farmed ungulate species, whereas few studies have been carried out in wild animal populations (Table S1). *Cryptosporidium* infections have been identified in wild ungulates of the genera *Capreolus*, *Cervus*, and *Sus* with prevalence rates ranging from 4~~–~~ to 17%. *Cryptosporidium bovis* and *C. ryanae* have been detected in roe deer (*Capreolus capreolus*), and *C. parvum*, *C suis*, and *C. scrofarum* in wild boar (Table S1). *Giardia duodenalis* has been reported in members of the genera *Capreolus*, *Cervus*, and *Sus* at prevalence rates of 1–23%. Only assemblage A has been documented in roe deer (García-Presedo et al., 2013a). A single study reported the presence of *B. coli* in wild boar in southern province of Córdoba (Rivero-Juarez et al., 2020). Given the scarcity of molecular epidemiological data on these enteric parasites in ungulate species in Spain, the purpose of the current study was to investigate the occurrence, genetic diversity, and zoonotic potential of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in free-ranging and farmed wild ungulates at the national scale.

~~2.~~2 M~~ATERIALS~~aterials ~~AND~~and ~~METHODS~~methods

~~2.1.~~2.1 Ethical statement

Sampled animals were legally hunted under Spanish (RD 8/2003, RD 138/2020) and EU (RD 53/2013) legislation. All the hunters had hunting licenses. Professional personnel collected the faecal samples from hunter-harvested wild ruminants and boars during the regular hunting seasons comprising from October to February.

~~2.2.~~2.2 Study area and sampling strategy

Between 1999 and 2021, a retrospective nationwide survey was performed. Faecal samples from the eight wild ungulate species present in Spain: Barbary sheep (*Ammotragus lervia*), Iberian wild goat (*Capra pyrenaica*), roe deer, red deer (*Cervus elaphus*), fallow deer (*Dama dama*), mouflon (*Ovis aries musimon*), southern chamois (*Rupicapra pyrenaica*), and wild boar, were collected throughout the five bioregions (BRs, see below) of mainland Spain (Table 1).

alt-text: Table 1

~~TABLE 1.~~Table 1

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Infection rates of the protozoan intestinal parasites investigated in the present survey according to host species.

Host species	Samples (*n*)	*G. duodenalis* % (positive/total)	*Cryptosporidium* spp. % (positive/total)	*B. coli* % (positive/total)
Barbary sheep	20	0.0 (0/20)	0.0 (0/20)	0.0 (0/20)
Fallow deer	96	5.2 (5/96)	0.0 (0/96)	0.0 (0/96)
Mouflon	10	10.0 (1/10)	0.0 (0/10)	0.0 (0/10)
Red deer[a]	653	3.8 (25/653)	1.5 (10/653)	0.0 (0/653)
Roe deer	93	7.5 (7/93)	7.5 (7/93)	0.0 (0/93)
Southern chamois	62	12.9 (8/62)	0.0 (0/62)	0.0 (0/62)
Iberian wild goat	89	9.0 (8/89)	0.0 (0/89)	0.0 (0/89)
Wild boar	359	5.6 (20/359)	7.0 (25/359)	2.5 (9/359)
Total	1~~-~~382	5.4 (74/1~~-~~382)	3.0 (42/1~~-~~382)	0.7 (9/1~~-~~382)

Table Footnotes

[a] Including farmed red deer (n = 324) and wild red deer (n = 329).

Based on landscape structure, major ecosystems, game management practices, and socio-political aspects, the Spanish Wildlife Disease Surveillance Scheme splits mainland Spain into five different BRs sharing similar epidemiological features (PNVSFS, 2020). BR1 comprises the Northern areas of temperate Atlantic climate with almost no game management; meanwhile, the remaining BRs present a Mediterranean climate with an increasing drought gradient from BR2 to BR4. In the Mediterranean BRs, game management is not the norm except for BR3 and the Southwest of BR5, where the highly productive savannah-like or oak forest landscapes are frequently profited for big-game hunting. Mountain habitats are more dominant in BRs 1, 2, and 5, while cereal plains are predominant in BR4. This zoning has been previously exploited to facilitate disease surveillance efforts in wild ungulates in Spain (García-Bocanegra et al., 2016; González-Barrio et al., 2015; Jiménez-Ruiz et al., 2021; Lorca-Oró et al., 2014; Muñoz et al., 2010). From each sampling site, that is, hunting estates or game reserves (n = 63, Table S2) selected by simple random sampling throughout the study area, the animals (15–20 whenever possible) were also randomly sampled. Fig. 1 shows the map of the Iberian Peninsula showing the sampling areas and the geographical distribution of protozoan DNA detected according to the bioregion of origin in all free-ranging and farmed wild ungulate species (Panel A), in free-ranging and farmed wild cervid (fallow deer, red deer, and roe deer) species only (Panel B), in free-ranging wild bovid (Barbary sheep, Iberian wild goat, mouflon, and southern chamois) species only (Panel C), and in wild boar only (Panel D).

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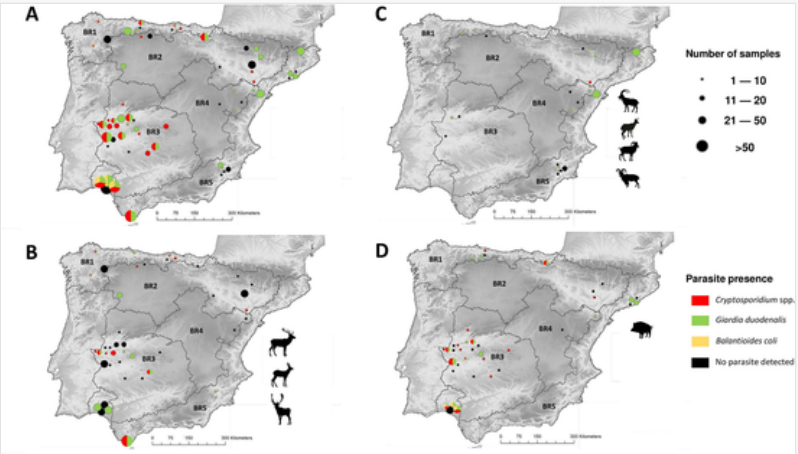
~~FIG. 1~~Fig. 1



Map of the Iberian Peninsula showing the sampling areas and the geographical distribution of protozoan DNA detected according to established bioregions (BR1–5) in Spain (Muñoz et al., 2010). Coloured circles indicate the presence of the protozoan species (red: *Cryptosporidium* spp.; green: *G. duodenalis*; yellow: *Balantioides coli*) investigated in each sampling area. Black filled circles indicate a negative PCR result. The area of the represented circles is proportional to the sample size (see figure legend). Panel A: all free-ranging and farmed wild ungulate species. Panel B: Free-ranging and farmed wild cervid species only. Panel C: Free-ranging wild bovid species only. Panel D: Free-ranging wild boar only. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Faecal samples were collected directly from the rectum of each animal during field necropsies after hunting using disposable gloves and placed in individual sterile tubes with records of the date, location, and host species. Collected samples were transported in cooled boxes to each participating institution responsible for the sampling and stored at –

−20 °C. Aliquots of these faecal samples were shipped to the Spanish National Centre for Microbiology, Majadahonda (Spain) for subsequent molecular analyses.

For comparative purposes, aliquots of faecal samples from a farmed wild red deer population located in southern Spain (Fig. 1, Panel B) were obtained from previous work (González-Barrio et al., 2017). These red deer were semi-extensively bred in a forest-shrub prairie habitat divided into different plots by high-wire fencing. The animals were kept in separate batches according to their sex and productive status. Batches of 60–80 reproductive females were kept within large-fenced areas (6–8 ha), whereas males were kept in separate enclosures. The animals were identified with individual ear tags. Faecal material was collected directly from the rectum using sterile disposable latex gloves during routine health veterinary inspections.

~~2.3.~~2.3 DNA extraction and purification

Genomic DNA was isolated from about 200 mg of each faecal specimen of free-ranging or farmed wild ungulate by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer~~'~~s instructions, except that samples mixed with InhibitEX buffer were incubated for 10 min at 95 °C. Extracted and purified DNA samples were eluted in 200 µl of PCR-grade water and kept at 4 °C until further molecular analysis.

~~2.4.~~2.4 Molecular detection and ~~characterisation~~characterization of *Cryptosporidium* spp., *Giardia duodenalis,* and *Balantioides coli*

Detailed information on the PCR cycling conditions and oligonucleotides used for the molecular identification and/or ~~characterisation~~characterization of the protozoan parasites investigated in the present study is presented in Tables S3 and S4, respectively.

The presence of *Cryptosporidium* spp. were assessed using a nested PCR protocol to amplify a 587-bp fragment of the small subunit of the rRNA (*ssu* rRNA) gene of the parasite (Tiangtip and Jongwutiwes, 2002). Specific subtyping tools targeting the partial 60-kDa glycoprotein gene (*gp60*) were used in those samples that tested positive for Q5 *Cryptosporidium* spp. by *ssu*-PCR including *C. canis* (Jiang et al., 2021), *C. parvum* (Feltus et al., 2006), *C. ryanae* (Yang et al., 2020), and *C. ubiquitum* (Li et al., 2014) to ascertain intra-species genetic diversity.

The presence of *G. duodenalis* was investigated using a real-time PCR (qPCR) method targeting a 62-bp region of the *ssu* rRNA gene of the parasite as the initial screening method (Verweij et al., 2003). For assessing the molecular diversity of *G. duodenalis* at the assemblage level, a nested PCR was used to amplify a 300-bp fragment of the *ssu* rRNA gene in those samples that yielded cycle threshold (C_T) values <35 in qPCR (Appelbee et al., 2003; Hopkins et al., 1997~~Hopkins et al., 1997~~). For assessing the molecular diversity of the parasite at the sub-assemblage level we adopted a sequence-based multilocus genotyping (MLST) scheme targeting the genes encoding for the glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. Only samples that yielded qPCR C_T values <32 were assessed under the MLST scheme. A semi-nested PCR was used to amplify a 432-bp fragment of the *gdh* gene (Read et al., 2004), and nested PCRs were used to amplify 511 and 530 bp fragments of the *bg* and *tpi* genes, respectively (Lalle et al., 2005; Sulaiman et al., 2003).

Balantioides coli detection was attempted by a direct PCR assay to amplify the complete ITS1–5.8 s-rRNA–ITS2 region and the last 117 bp (3′ end) of the *ssu*-rRNA sequence of this ciliate using previously published procedures (Ponce-Gordo et al., 2011).

All the direct, semi-nested and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always included 2.5 U of MyTAQ~~™~~™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany) and 5–10 µl 5× MyTAQ~~™~~™ Reaction Buffer containing 5 mM deoxynucleotide triphosphates and 15 mM $MgCl_2$. Negative and positive controls were included in every PCR run. PCR amplicons were visualized on 2% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe (Conda nucleic acid staining solutions. A 100 bp DNA ladder (Boehringer Mannheim GmbH, Mannheim, Germany) was used for the sizing of the obtained amplicons.

~~2.5.~~2.5 Sequence analysis

All amplicons of the expected size were directly sequenced in both directions with the corresponding internal primer pair (see Table S4) in 10 µl reactions using Big Dye~~™~~™ chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA). Raw sequences were examined with Chromas Lite version 2.1 software (http://chromaslite.software.informer.com/2.1) to generate consensus sequences. These sequences were compared with reference sequences deposited at the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences generated in the present study were deposited in the GenBank public repository database under accession numbers OP164760 (*C. canis*), OP164761–OP164762 (*C. parvum*), OP164763–OP164767 (*C. ryanae*), OP164768–OP164773 (*C. scrofarum*), OP164774 (*C. suis*), OP164775 (*C. ubiquitum*), and OP888101-OP888103 (*G. duodenalis*).

~~2.6.~~2.6 Statistical analysis

To explore whether bioregions have specific pathogen communities we use a Permutational Multivariate Analysis of Variance (PERMANOVA). Two fixed factors were entered into the analysis: bioregion (with five levels) and species (with eight levels). Type III Sum of Squares was used since it is appropriate in the case of an unbalanced design. All the tests were performed with 999 permutations to increase the power and precision of analysis (Anderson et al., 2008) of residuals under a reduced model (Anderson and Braak, 2003). The differences in community structure among the bioregion and species were investigated using a posteriori pair-wise test with 999 permutations. The advantage of the permutation approach is that the resulting test is "distribution-free" and not constrained by many of the typical assumptions of parametric statistics (Walters and Coen, 2006). The relative contribution of each pathogen species to determine differences in the composition of the communities between bioregion and species were evaluated using a similarity percentage analysis (SIMPER). In this study, SIMPER was employed to identify those pathogen species that were responsible for ~~more than~~ ≥90% of dissimilarity among bioregion and species. All the analyses were performed using PRIMER v6 software (Clarke and Gorley, 2006), including the PERMANOVA+ add-on package (Anderson et al., 2008).

~~3.~~3 R~~ESULTS~~esults

~~3.1.~~3.1 Occurrence of enteric protozoa

The full dataset of this study showing sampling, diagnostic, and molecular data can be found in Table S5. A total of 1~~,~~382 samples from free-ranging ungulates (76.6%, 1058/1~~,~~382) and farmed wild red deer (23.4%, 324/1~~,~~382) were included in the survey. Overall, *G. duodenalis* was the most prevalent enteric parasite found in the wild and farmed ungulate species (5.4%, 74/1~~,~~382; 95% CI: 4.2–6.5%), followed by *Cryptosporidium* spp. (3.0%, 42/1~~,~~382; 95% CI: 2.1–3.9%), and *B. coli* (0.7%, 9/1~~,~~382; 95% CI: 0.3–1.2%).

Cryptosporidium infection was only detected in roe deer (7.5%, 7/93), wild boar (7.0%, 25/359) and red deer (1.5%, 10/653). This protozoan was detected in BR3 (7.5%, 25/335), BR1 (6.8%, 7/103), BR2 (4.3%, 7/164) and BR5 (0.4%, 3/748) (Table 1, Fig. 1). Unlike the Barbary sheep (n = 20), *G. duodenalis* was detected in seven of the studied host species including southern chamois (12.9%, 8/62), mouflon (10.0%, 1/10), Iberian wild goat (9.0%, 8/89), roe deer (7.5%, 7/93), wild boar (5.6%, 20/359), fallow deer (5.2%, 5/96) and red deer (3.8%, 25/653) (Fig. 1, Table 1). The infection rate of *G. duodenalis* detected in farmed wild red deer (5.2%, 17/324) was higher than in free-ranging red deer (2.4%, 8/329). According to the spatial distribution in the bioregion of origin, *G. duodenalis* infections were more frequent in BR1 (12.6%, 13/103) than in BR4 (12.5%, 4/32), BR5 (4.9%, 37/748), BR2 (4.3%, 7/164) and BR3 (3.9%, 13/335). The ciliate *B. coli* was only detected in wild boar (2.5%, 9/359) from the BR5 (1.2%, 9/748) (Fig. 1, Table 1).

~~3.2.~~3.2 Molecular diversity

Six *Cryptosporidium* species were identified in the ungulate population investigated: *C. scrofarum* (52.4%, 22/42), *C. ryanae* (31.0%. 13/42), *C. parvum* (7.1%, 3/42), *C. canis* (2.4%, 1/42), *C. suis* (2.4%, 1/42), and *C. ubiquitum* (2.4%, 1/42) (Table 2). *Cryptosporidium scrofarum* was identified exclusively in wild boar, whereas *C. ryanae* was found infecting red deer (n = 7), roe deer (n = 5), and wild boar (n = 1). *Cryptosporidium parvum* was detected in red deer (n = 2) and wild boar (n = 1), *C. ubiquitum* and canine-adapted *C. canis* in roe deer (one each), and swine-adapted *C. suis* in red deer. An additional wild boar sample (very likely belonging to *C. scrofarum*) could not be assigned to a given *Cryptosporidium* species due to insufficient sequence quality. *Cryptosporidium scrofarum*, *C. ryanae,* and *C. parvum* had a wide geographical distribution, being identified in two or more bioregions. *Cryptosporidium suis* was only detected in BR3, *C. canis* in BR2, and *C. ubiquitum* in BR1 (Table S5).

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~~Table 2.~~Table 2

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Frequency and molecular diversity of *Cryptosporidium* spp. and *G. duodenalis* identified at the *ssu* rRNA gene in the free-ranging and farmed wild ungulate species investigated in the present study.

Species	Sub-genotype	No. isolates	Reference sequence	Stretch	Single nucleotide polymorphisms	GenBank ID
C. canis	Unknown	1	AF112576	528–976	None	OP164760
C. parvum	Unknown	2	AF112571	527–1~~,~~026	A646G, T649G, 686_689DelTAAT,	OP164761

					T693A	
		1	AF112571	528–1030	A646G, T649G, 686_689DelTAAT, A691T, T952Y	OP164762
C. ryanae	Unknown	9	MK982509	339–829	None	OP164763
	Unknown	1	MK982509	345–828	C448Y	OP164764
	Unknown	1	MK982509	388–818	C448T, C507T	OP164765
	Unknown	1	MK982509	336–828	A452G, 496InsA	OP164766
	Unknown	1	MK982509	345–828	A479G	OP164767
C. scrofarum	–	16	KF597534	284–734	None	OP164768
	–	2	KF597534	293–734	A441R	OP164769
	–	1	KF597534	282–734	A424G, C616Y, A669G	OP164770
	–	1	KF597534	296–734	T432C, A441G	OP164771
	–	1	KF597534	281–730	438InsTT	OP164772
	–	1	KF597534	281–734	C616Y	OP164773
C. suis	–	1	AF115377	643–945	None	OP164774
C. ubiquitum	Unknown	1	MH059802	357–837	498Ins_AT	OP164775
C. spp.[a]	Unknown	1	–	–	–	–
G. duodenalis	A	1	AF199446	3–292	None	OP888101
	B	1	AF199447	5–223	None	OP888102
	E	4	AF199448	1–292	None	OP888103

Table Footnotes

[a] Sequence of insufficient quality to accurately ascertain the *Cryptosporidium* species involved.

None of the *Cryptosporidium*-positive samples could be subtyped at the *gp60* gene. Remarkably, the *ssu*-PCR used for initial *Cryptosporidium* detection yielded many unspecific amplification reactions (n = 113). Sanger sequencing analysis of these products revealed the presence of bacterial (Aeromonas), fungal (genera *Alternaria*, *Bipolaris*, *Cercophora*, *Fusarium*, *Gnomoniopsis*, *Preussia*, *Sirococcus*, *Sordaria*, *Thelebolus*), plant (genera *Fagus*, *Pelargonium*), algae (genus *Desmodesmus*) or other protist (genera *Adelina*, *Colpodella*, *Cyclotella*, *Platyophrides*, *Stylonychia*, *Theileria*) organisms (Table S5).

Giardia duodenalis-positive samples from free-ranging and farmed wild ungulate species (n = 74) generated cycle threshold (C_T) values ranging from 20.9 to 39.5 (median: 33.7; SD: 3.6). To maximize resources and time, only samples with C_T values ≤35 (n = 35) were attempted to be genotyped at the *ssu* rRNA locus for assemblage determination. Sequence analyses revealed the presence of zoonotic assemblages A (n = 1) and B (n = 1) and ungulate-adapted assemblage E (n = 4) (Table 2). Additionally, samples with C_T values ≤32 (n = 21) were assessed at the *gdh*, *bg*, and *tpi* loci for sub-assemblage determination, but none of them could be successfully amplified at these makers.

Balantioides coli was unmistakably identified in eight wild boar samples, but sequence data of insufficient quality precluded the possibility of determining the genotype of this parasite species. Four additional amplicons (also from swine origin) yielded faint bands on gel electrophoresis that could not be confirmed by Sanger sequencing. These four samples were conservatively regarded as *B. coli*-negative.

~~3.3.~~3.3 Pathogen community

The PERMANOVA analysis revealed significant differences in the composition of pathogen species across bioregion (P = 0.001) and host species (P = 0.002). Pair-wise test results revealed significant differences in pathogen compositions according to bioregions (between BR2 vs. BR1, BR2 vs. BR5, and BR3 vs. BR5) and host species (between *C. elaphus* vs. *S. scrofa* and *C. elaphus* vs. *R. pyrenaica* (Table 3).

alt-text: Table 3

~~Table 3.~~Table 3

Results of multivariate PERMANOVA (A) main test and pairwise tests assessing for the presence/absence of pathogens for each pair of bioregions (B) and host species (C). *P*-values in bold indicate statistical significance.

A. PERMANOVA test						
Variable	*Df*	*SSq*	*MS*	*Pseudo-F*	*P*	*Perms*
Bioregion	4	1930	482	4.89	**0.001**	998
Host species	7	1698	242	2.45	**0.002**	998
Res	1370	1.35E5	98.7			
Total	1381	1.39E5				

B. Pair-wise test (according to bioregion)			
Groups	*t*	*P*	*Perms*
BR2, BR1	1.95	**0.03**	999
BR2, BR3	0.35	0.832	998
BR2, BR5	2.01	**0.021**	998
BR2, BR4	1.04	0.289	997
BR1, BR3	1.28	0.206	999
BR1, BR5	1.64	0.091	999
BR1, BR4	0.89	0.425	997
BR3, BR5	3.55	**0.001**	999
BR3, BR4	1.23	0.17	999
BR5, BR4	1.63	0.077	999

C. Pair-wise test (according to host species)			
Groups	*t*	*P*	*Perms*
C.e., C.c.	1.47	0.146	999
C.e., S.s.	3.09	**0.001**	999
C.e., R.p.	2.73	**0.003**	999
C.e., D.d.	0.48	0.764	999
C.e., O.a.m.	1.06	0.243	999
C.e., C.p.	0.94	0.393	999
C.e., A.l.	0.83	0.43	999
C.c., S.s.	Negative	–	–
C.c., R.p.	1.44	0.137	999
C.c., D.d.	1.36	0.143	999
C.c., O.a.m.	No test (df = 0)	–	–
C.c., C.p.	0.80	0.422	999
C.c., A.l.	No test (df = 0)	–	–
S.s., R.p.	1.58	0.081	998
S.s., D.d.	1.28	0.192	999
S.s., O.a.m.	1.04	0.257	998
S.s., C.p.	1.23	0.212	998
S.s., A.l.	0.81	0.547	998
R.p., D.d.	1.70	0.093	997

R.p., O.a.m.	0.35	0.474	995
R.p., C.p.	0.69	0.505	998
R.p., A.l.	1.36	0.194	994
D.d., O.a.m.	0.74	0.403	962
D.d., C.p.	0.49	0.625	998
D.d., A.l.	0.99	0.439	919
O.a.m., C.p.	0.24	0.64	984
O.a.m., A.l.	Negative	–	–
C.p., A.l.	1.09	0.284	770

A.l.: *Ammotragus lervia*; C.c.: *Capreolus capreolus*; C.e.: *Cervus elaphus*; C.p.: *Capra pyrenaica*; D.d.: *Dama dama*; Df: degrees of freedom; MS: mean sum of squares; O.a.m.: *Ovis aries musimon*; Pseudo-F: F value by permutation; R.p. *Rupicabra pyrenaica*; S.s.: *Sus scrofa*; SSq: sum of squares.

The SIMPER analysis showed considerable average dissimilarity between bioregions: BR2 vs. BR1 (96.8%), BR2 vs. BR5 (98.4%), and BR3 vs. BR5 (98.7%). The species with the highest dissimilarity contribution between bioregions was *G. duodenalis* (Table 4). The SIMPER analysis also showed considerable average dissimilarity between species: *C. elaphus* vs. *Sus scrofa* (98.5%) and *C. elaphus* vs. *R. pyrenaica* (97.2%). The species with the highest dissimilarity contribution values between host species also was *G. duodenalis*.

alt-text: Table 4

Table 4

SIMPER results of pathogen species that contribute at dissimilarity between bioregions and host species.

Species	Contribution %	Cumulative %
Dissimilarity between BR1 vs. BR2: 96.8%		
Giardia duodenalis	61.64	61.34
Cryptosporidium spp.	38.36	100
Dissimilarity between BR2 vs. BR5: 98.4%		
Giardia duodenalis	60.51	60.51
Cryptosporidium spp.	31.44	100
Dissimilarity between BR3 vs. BR5: 98.7%		
Giardia duodenalis	46.86	46.86
Cryptosporidium spp.	40.98	87.84
Balantioides coli	12.16	100
Dissimilarity between C.e. vs. S.s.: 98.5%		
Giardia duodenalis	43.85	43.85
Cryptosporidium spp.	38.92	82.77
Balantioides coli	17.23	100
Dissimilarity between C.e. vs. R.p.: 97.2%		
Giardia duodenalis	91.96	91.96
Cryptosporidium spp.	8.04	100

C.e.: *Cervus elaphus*; R.p. *Rupicabra pyrenaica*; S.s.: *Sus scrofa*.

~~4.~~4 D~~ISCUSSION~~iscussion

This study represents the largest attempt to assess the occurrence, molecular diversity, and zoonotic potential of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in wild hoofed animals conducted in Spain to date. A molecular-based study specifically devoted to the Microsporidia *Enterocytozoon bieneusi* was previously published in this very same population (Dashti et al., 2022). Our study had several strengths, including a large sample size, representativeness of all eight wild ungulate species present in the country, national coverage, and molecular-based diagnosis and genotyping approaches. The survey is also timely because information on the wild ungulate contribution to environmental *Cryptosporidium* oocysts and *Giardia* cysts is scarce (Hatam-Nahavandi et al., 2019). Regarding *B. coli*, little is known about the role of non-swine species as potentially suitable hosts for this ciliate parasite (Ponce-Gordo and García-Rodríguez, 2021).

Our data revealed an overall *Cryptosporidium* infection rate of 3%, peaking at 7–8% in roe deer and wild boar, respectively. These figures agree with that (8%, 484/9~~,~~480) estimated in a recent systematic review and meta-analysis of *Cryptosporidium* infection prevalence in deer worldwide (Lv et al., 2021). In Spain, most of the epidemiological studies on protozoan infections in domestic and wild ruminants have been conducted in Galicia (north-western areas of the country) (see Table S1). In this region *Cryptosporidium* spp. infections have been reported in 7% of red deer, in 1–4% of roe deer, and in 7–17% of wild boar (Castro-Hermida et al., 2011a, 2011b; García-Presedo et al., 2013a, 2013b). A prevalence rate of 6% has also been documented in wild boar in the south of Spain (Rivero-Juarez et al., 2020). At the European scenario, *Cryptosporidium* spp. infections have been identified in nine different species of wild ungulate species. These include 2% of Alpine chamois (*Rupicapra rupicapra*) in Italy (Trogu et al., 2021), 6% of fallow deer in the UK (Sturdee et al., 1999), 3% of moose (*Alces alces*) in Norway (Hamnes et al., 2006), 2% of mouflons (*Ovis musimon*) in the Czech Republic (Kotkova et al., 2016), 0.3–80% of red deer in the Czech Republic (Kotkova et al., 2016), Ireland (Skerrett and Holland, 2001), Italy (Trogu et al., 2021), Poland (Paziewska et al., 2007), Norway (Hamnes et al., 2006), and UK (Wells et al., 2015), 10% of Reeve~~'~~s muntjac (*Muntiacus reevesi*) in the UK (Sturdee et al., 1999), 3–33% of roe deer in Italy (Trogu et al., 2021), Poland (Paziewska et al., 2007), and Norway (Hamnes et al., 2006), 12% of white-tailed deer (*Odocoileus virginianus*) in the Czech Republic (Kotkova et al., 2016), and 13–17% of wild boar in central Europe (Němejc et al., 2012, 2013).

Our sequence analyses confirmed the occurrence of six distinct *Cryptosporidium* species circulating within the surveyed wild ungulate population. Of them, *C. ryanae* showed the widest host range, being detected in red deer, roe deer, and wild boar. *Cryptosporidium parvum* was identified in red deer and wild boar, but less frequently than *C. ryanae*. *Cryptosporidium ubiquitum* was observed only in one roe deer sample, whereas *C. scrofarum* (formerly known as pig genotype II) was exclusively detected in wild boar. The findings of *C. canis* in one roe deer and *C. suis* in one red deer are interesting. These *Cryptosporidium* species are adapted to infect canids and swine, respectively, suggesting that their presence in wild cervids could be the result of overlapping sylvatic and/or domestic transmission cycles of the parasite in habitats where different host species live sympatrically. Whether these findings correspond to true or spurious (mechanical carriage) infections remain to be elucidated.

Only a single study has previously reported molecular data on wild cervids in Spain, showing the occurrence of *C. ryanae* and *C. bovis* infections in roe deer in Galicia (García-Presedo et al., 2013a). At the European level, *C. ubiquitum* is also the predominant *Cryptosporidium* species circulating in wild ruminants, being identified in red deer in the Czech Republic (Kotkova et al., 2016), roe deer in Italy and the UK (Trogu et al., 2021), and Alpine chamois in Italy (Trogu et al., 2021). *Cryptosporidium* deer genotype has also been reported in white-tailed deer in the Czech Republic (Kotkova et al., 2016) and in red deer and roe deer in the UK (Robinson et al., 2011; Wells et al., 2015), whereas sporadic cases of infections by murine-adapted *C. muris* have been identified in mouflon, red deer and white-tailed deer (one each) in the Czech Republic (Kotkova et al., 2016). Neither *C.* deer genotype and *C. muris* nor *C. bovis* were identified in the Spanish population surveyed in the present study. Remarkably, *C. parvum* is a common finding in wild cervids in the UK (Wells et al., 2015), likely as a result of spill over events from infected livestock. Regarding wild boar, both *C. scrofarum* and *C. suis* have been documented at similar proportions in Central European countries including Austria, Czech Republic, Poland, and Slovakia (Němejc et al., 2012, 2013). This is not the case in Spain, where *C. scrofarum* is far more prevalent than *C. suis* in this host species (Rivero-Juarez et al., 2020; present study).

In our study, *G. duodenalis* was the most prevalent protozoan parasite found (5%), with peaks of 10–13% in mouflons and southern chamois. In Spain, this parasite has been previously identified at infection rates of 8% in red deer, 5–9% in roe deer, and 1–4% in wild boar in Galicia (Castro-Hermida et al., 2011a, 2011b; García-Presedo et al., 2013a). A higher infection rate of 23% was documented in wild boar in the southern province of Córdoba (Rivero-Juarez et al., 2020). In other European countries, *G. duodenalis* has been described in six genera of wild ungulates including *Alces*, *Capreolus*, *Cervus*, *Dama*, *Rangifer*, and *Sus*. Prevalences of 1–2% and 4–24% have been obtained in red deer and roe deer, respectively, in Croatia (Beck et al., 2011), Norway (Hamnes et al., 2006), and Poland (Majewska et al., 2012; Paziewska et al., 2007; Solarczyk et al., 2012~~Solarczyk et al., 2012~~). *Giardia duodenalis* infections have also been

documented at rates of 12% in fallow deer in Italy (Lalle et al., 2007), of 5–12% in moose and reindeer (*Rangifer tarandus*) in Norway (Hamnes et al., 2006; Robertson et al., 2007), and in 2–4% in wild boar in Croatia and Switzerland (Beck et al., 2011; Spieler and Schnyder, 2021).

Ssu rRNA sequence analyses revealed the presence of zoonotic assemblages A and B in single samples from wild boar and red deer, whereas ungulate-adapted assemblage E was identified in mouflon, red deer, and southern chamois. We failed to amplify any of these isolates at the *gdh*, *bg* or *tpi* loci, so information at the sub-assemblage level is still lacking. Similar negative sub-genotyping results have been obtained in previous studies by our research group when investigating wild boar (Rivero-Juarez et al., 2020). Information on the molecular diversity of *G. duodenalis* in wild ungulate European populations is also limited. Assemblage A seems to be the most prevalent genetic variant of the parasite, being detected in red deer, roe deer, and wild boar in Croatia (Beck et al., 2011), in fallow deer in Italy (Lalle et al., 2007), in moose and reindeer in Norway (Idland et al., 2021; Robertson et al., 2007), and in roe deer in Spain (García-Presedo et al., 2013a). Interestingly, canine-adapted *G. duodenalis* assemblages C/D and murine-adapted *G. muris* have been sporadically detected in Croatian roe deer and wild boar (Beck et al., 2011), indicative of cross-species transmission or overlapping of transmission cycles of these pathogens. As in the case of *C. canis* and *C. suis*, at present it is unclear whether these findings correspond to true or spurious infections.

Swine-adapted *B. coli* was identified at low frequency (<3%) in wild boar only. This finding suggests that wild ruminants are not suitable hosts for this ciliate parasite, which in Europe seems naturally restricted to pigs and, to a lesser extent, wild boar. A *B. coli* infection rate of 2% has been previously reported in wild boar in Switzerland (Spieler and Schnyder, 2021).

Our study provided also interesting data about differences in protozoan communities among bioregions. Because higher interaction rates (facilitating pathogen transmission) are more likely to occur among animals living in restricted areas than among wandering animals, one would expect that wild farmed red deer would bear higher protozoan infection rates than their free-ranging wild counterparts. Although this was the case for *G. duodenalis* infections (2.4% vs. 5.2%), exactly the opposite trend was observed for *Cryptosporidium* infections (2.7% vs. 0.3%). Interestingly, free-living wild red deer were infected by a higher diversity of *Cryptosporidium* species (*C. ryanae*, *C. parvum*, *C. suis*) than farmed wild red deer (in which only *C. parvum* was found), suggesting that the former were exposed to a wider range of infective sources. Regarding bioregion of origin, wild boar living in BR5 were less likely to be infected by *Cryptosporidium* spp., but not by *G. duodenalis*. A potential explanation for this finding is that BR5 comprises all the Spanish Mediterranean littoral, highly developed and urbanized and, at least in theory, less suitable for sustaining large populations of wild animals. Remarkably, all the *B. coli*-positive samples were collected in the Doñana National Park, a natural reserve extending over three provinces (Huelva, Cádiz, and Seville) in southern Spain, BR5. At present we do not have a clear explanation for the apparent geographical segregation observed for *B. coli* in wild boar.

This study had four potential limitations that may have biased the results obtained. First, its retroactive nature implied that some of the faecal samples analysed were stored at ~~–~~20 °C for up to 12 years prior DNA extraction and molecular testing. Long-term storage may have altered the quantity/quality of parasite DNA, compromising the performance of the PCRs used. Second, sample size for some wild ungulate species (e.g., mouflon and Barbary sheep) may be underrepresented. Of note, the natural populations of these ruminants in Spain are relatively low in numbers (mouflon: near 15,000 individuals; Barbary sheep: near 1~~-~~300 individuals) making difficult obtaining representative sample numbers. Third, the genetic markers used in our genotyping PCR protocols had intrinsic limited sensitivities. In practical terms this means that samples with little amount of parasite DNA would be amplified by the detection PCRs (targeting highly sensitive, multi-copy genes such as *ssu* rRNA) but not by the genotyping or subtyping PCRs (targeting low sensitive, single-copy genes such as *gdh*, *bg*, and *tpi* in *Giardia duodenalis* or *gp60* in *Cryptosporidium* spp.). Lack or insufficient genotyping data make difficult the assessment of zoonotic potential and the public health significance of the results obtained. Finally, direct comparison of results among bioregions should be interpreted with caution because of differences not only in environmental and epidemiological factors, but also in methodological approaches including sampling periods and strategies and diagnostic methods.

~~5.~~5 C~~ONCLUSIONS~~onclusions

This is the largest molecular epidemiological study investigating the presence and genetic diversity of *Cryptosporidium* spp., *G. duodenalis*, and *B. coli* in wild ungulate species conducted in Spain to date. Overall infection rates were relatively low (≤5%) and, in the case of *Cryptosporidium* and *Balantiodes* infections, mostly caused by ungulate-adapted species/genotypes. Sporadic infections by canine- or swine-adapted species may be indicative of potential cross-species transmission, although spurious infections cannot be ruled out. Taken together, these data would indicate that wild ungulate species pose a limited role as source of human infections by *Cryptosporidium* spp., *G. duodenalis*, or *B. coli*. Wild ruminants do not seem to be suitable hosts for *B. coli*, whose main ungulate host species is the wild boar.

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Wildlife Ecology & Health Group (WE&H)

The following investigators (in alphabetic order) participated in the Wildlife Ecology & Health Group (WE&H): Carles Conejero, Carlos González-Crespo, Cristina Garrido, Diana Gassó, Emmanuel Serrano, Gregorio Mentaberre, Helena Martínez-Torres, Irene Torres-Blas, Josep Estruch, Josep Pastor, Jorge Ramón López-Olvera, María Escobar-González, Marta Valldeperes, Montse Mesalles, Raquel Álvarez, Rafaela Cuenca, Roser Velarde, Santiago Lavín.

A~~UTHORS~~uthors ~~CONTRIBUTIONS~~Contributions

MAH, ARJ, JV, MCA, DFL, PM, JAA, AB, GAC, CMC, JAO, RCB, DGB and ES (on behalf of the WE&H group) collected the samples. AD, PCK, BB and ASM carried out the laboratory experiments. DC and DGB designed and supervised the experiments. AD, DGB and DC writing—original draft preparation. ARJ, AB, RCB, ES, DGB and DC writing—review and editing. The final version was read and approved by all authors.

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~~Hopkins et al., 1997~~

~~R Core Team, 2012~~

~~Solarczyk et al., 2012~~

Declaration of Competing ~~ONFLICTOFINTEREST~~Interest

The authors have declared no conflict of interest.

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Q7 interpretation of the study.

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Highlights

- The largest Spanish nationwide study to investigate microparasites in wild ungulates.
- Infection rates were relatively low and caused by ungulate-adapted species/genotypes.
- Sporadic infections by *Cryptosporidium*-adapted species may be indicative of potential cross-species transmission.
- Our molecular evidence is consistent with mild infections and limited environmental contamination with (oo)cysts.
- Wild ruminants are not suitable hosts for *B. coli*.

The following are the supplementary data related to this article.

Multimedia Component 1

Table S1

Infection rates and molecular diversity of *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* reported in wild and farmed ungulate (cervid and wild boar) species in Spain.

alt-text: Table S1

Multimedia Component 2

Table S2

Number and relative frequencies of faecal samples from free-ranging and farmed wild ungulates (n = 1,382) analysed in the present survey according to the bioregion of origin.

alt-text: Table S2

Multimedia Component 3

Table S3

PCR cycling conditions used for the molecular identification and/or characterization of the microeukaryotic parasites investigated in the present study.

alt-text: Table S3

Multimedia Component 4

Table S4

Oligonucleotides used for the molecular identification and/or characterization of *Cryptosporidium* spp., *Giardia duodenalis*, and *Balantioides coli* in the present study.

alt-text: Table S4

Multimedia Component 5

Table S5

Full dataset generated in the present study showing epidemiological, diagnostic, and genotyping results.

alt-text: Table S5

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Q1

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