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Prevalence and public health relevance of enteric parasites in domestic dogs and cats in the region of Madrid (Spain) with an emphasis on *Giardia duodenalis* and *Cryptosporidium* sp.

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Abstract

Background: Pet dogs and cats exert an unquestionable beneficial effect in the well-being of their owners, but can also act as a source of zoonotic infections if improperly cared.

Objectives: We investigated the occurrence, risk factors, genetic variability and zoonotic potential of intestinal parasites in dogs and cats attended in a clinical veterinary setting in Spain.

Methods: Canine (n = 252) and feline (n = 35) faecal samples were collected during 2017–2019 and analysed by coproparasitological methods. A rapid lateral immunochromatographic test (ICT) was used for detecting *Giardia duodenalis* and *Cryptosporidium* sp. Samples positive at microscopy examination and/or ICT were reassessed by molecular methods.

Results: Overall, 48.8% (123/252) of dogs and 48.6% (17/35) of cats were infected by enteric parasites. In dogs, *G. duodenalis* was the most prevalent species (40.9%), followed by *Cystoisospora* sp. (7.1%), and *Toxocara canis* (5.2%). In cats, *Joyeuxiella* sp. and *Toxocara cati* were the dominant species (20.0% each), followed by *G. duodenalis* (14.3%), *D. caninum* (5.7%) and *Cystoisospora felis* and *Toxascaris leonina* (2.9% each). Pups and kittens were more likely to harbour intestinal parasites and develop clinical signs. Sequence analyses of dog isolates revealed the presence of assemblages

A ($n = 1$), C ($n = 4$), D ($n = 4$) and C+D ($n = 1$) within *G. duodenalis*; *C. parvum* ($n = 1$) and *C. canis* ($n = 4$) within *Cryptosporidium* and PtEb IX ($n = 1$) in *Enterocytozoon bieneusi*. A novel *C. canis* subtype family, named XXi, is reported.

Conclusions: Our results highlight that (i) well-cared dogs carry zoonotic enteric protozoan parasites of public health relevance, (ii) proper hygiene practices and routine veterinary treatment are essential to prevent zoonotic infections, (iii) vulnerable populations should avoid contact with pups/kittens with diarrhoea and (iv) infected dogs might be major contributors to the environmental contamination with soil-transmitted helminths (STHs) eggs.

KEYWORDS
genotyping, helminths, prevention, protists, small animal clinic, transmission, zoonosis

1 | INTRODUCTION

Pet ownership can have a beneficial impact on human health, providing emotional, social and physical well-being benefits (Friedman & Krause-Parello, 2018). Interacting with dogs and cats has been demonstrated beneficial for improve mental health by reducing stress, anxiety and depression, and by promoting feelings of happiness and companionship (Boldig & Butala, 2021). Owning a pet also encourages physical activity and contributes to improved physical fitness and cardiovascular health (Arhant-Sudhir et al., 2011; Cutt et al., 2007). However, pet owning comes with the potential for zoonotic infections (Baneth et al., 2016). Domestic dogs and cats can carry a large variety of bacterial, viral and parasitic pathogens which can be transmitted to humans through bites, scratches, saliva, urine, faeces or contaminated surfaces (Overgaauw et al., 2020). Young children, pregnant women, elderly individuals and immunocompromised individuals might be more susceptible to zoonotic infections (Meers et al., 2022). In addition to adequate hygiene practices (e.g. regular handwashing, proper handling and disposal of dog waste), routine veterinary care is essential to prevent the transmission of zoonotic diseases and ensure the health and safety of dogs and their owners (ESCCAP, 2018, 2021; Miró et al., 2020; Morelli et al., 2022).

Among parasites, enteric protists and helminths are significant causes of morbidity and mortality in dogs and cats, particularly pups and kittens, senior animals or those with weakened immune systems (Gorgani-Firouzjaee et al., 2022; Raza et al., 2018; Traversa, 2012). These parasites include helminths (e.g. members of the families Ancylostomatidae, Dipylididae, Taeniidae, *Toxocara canis*, *Toxocara cati*, *Toxascaris leonina*, *Strongyloides* sp. and *Trichuris vulpis*) as well as protists (e.g. *Giardia duodenalis*, *Cryptosporidium* sp., *Cystoisospora* sp.), and are significant causes of diarrhoea with subsequent dehydration, weight loss, abdominal pain and occasionally, anaemia (Miller, 2020; Scorza & Tangtrongsup, 2010; Tangtrongsup & Scorza, 2010; Tysnes et al., 2014).

Regular anti-parasitic treatment of dogs is the most effective measure to minimise the risk of infection and transmission of zoonotic diseases. However, the commonly used anti-parasitic treatment used in the clinical practice consists in the administration of anthelmintic drugs, known to be ineffective against infections caused by protist parasites including *G. duodenalis* and *Cryptosporidium* sp. (ESCCAP 2018, 2021). Furthermore, prescribed treatments are generic and do not take into consideration epidemiological risk factors such as the likelihood of reinfections or the genetic variants of the parasite involved in the infection (Bagster & Elsheikha, 2022; McNamara et al., 2018). Furthermore, the complex transmission cycles of parasitic helminths, which include multiple hosts and life stages, often pose significant challenges for treatment efforts, requiring specific dosages and active drugs for effective eradication (Mengarda et al., 2023). Considering all the stated above, regular coproparasitological examination of canine and feline faecal samples should be carried out to monitor and evaluate the efficacy of the prescribed treatments.

This study aims at investing the occurrence of enteric helminthic and protist parasites in dogs and cats attended in a small animal clinic setting in central Spain, highlighting the importance of conducting coproparasitological analyses for prompt treatment and reducing the likelihood of zoonotic transmission events. Additional molecular studies were conducted to investigate the frequency and diversity of genetic variants of *G. duodenalis* and *Cryptosporidium* sp. circulating in the surveyed dog population.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003) and with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013).

2.2 | Study design and setting

This is an observational, retrospective epidemiological study summarising the results of the coproparasitological analyses conducted

in faecal samples from dogs (n = 252) and cats (n = 35) attended in a small animal veterinary clinic in the Majadahonda municipality (northwest Madrid) from January 2017 to December 2021. Majadahonda has 72,179 inhabitants and 3482 cats and 10,366 dogs censed in 2022 (Instituto Nacional de Estadística, 2022; RIAC). It is one of the Spaint's wealthiest municipalities ranking eight in average annual income per person and household (Instituto Nacional de Estadística, 2020). A total of 10 veterinary clinics and a large veterinary hospital provide health care services to the canine and feline populations in the local community.

2.3 | Microscopy detection

Three consecutive faecal samples from each investigated dog or cat were collected by pet owners after spontaneous defecation of their animals. Faecal matter was transferred into sterile polystyrene plastic flasks and kept at 4°C until further processing at the veterinary clinic. The cohort included animals of all age groups and breeds with and without gastrointestinal or respiratory disease manifestations. Dogs and cats with a previous diagnosis for one or more enteric parasites undergoing treatment follow-up were excluded from the study.

Intestinal parasites were diagnosed by the microscopic detection of their developmental stages (larvae, eggs, cysts, oocysts and trophozoites) in the faecal material using the modified Telemann concentration technique coupled with merthiolate-iodine-formaldehyde (MIF) staining (De Rivas, 1928; Telemann, 1908). No modified Ziehl–Neelsen staining for the detection of coccidian protozoa (e.g. *Cryptosporidium* sp.) in faecal smears was conducted. Faecal samples from dogs or cats with respiratory symptoms were analysed using the Baermann method for the specific detection of larvae of *Strongyloides* sp. and lungworms (ESCCAP, 2022).

2.4 | Immunochromatographic rapid test

All canine and feline faecal samples were investigated by an immunochromatographic rapid test (immunochromatographic test (ICT), Stick Crypto-Giardia, Operon, Zaragoza, Spain) for the simultaneous detection of the protozoa *G. duodenalis* and *Cryptosporidium* sp. following the manufacturert's instructions.

2.5 | DNA extraction and purification

Faecal concentrates that tested positive or dubious for enteric parasites by microscopy (*G. duodenalis*, *Strongyloides* sp.) or ICT (*G. duodenalis*, *Cryptosporidium* sp.) were stored for up to 3 months at −20°C and subsequently shipped to the Parasitology Reference and Research Laboratory, National Centre for Microbiology, Majadahonda (Madrid), for downstream molecular testing. Genomic DNA was isolated from about 200 mg of each concentrated faecal sample using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 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 for up to 6 months until further PCR analysis.

2.6 | Molecular detection and characterisation of *Giardia duodenalis*

Detection of *G. duodenalis* DNA was achieved using a real-time PCR (qPCR) method targeting the gene codifying the small subunit ribosomal RNA (*ssu* rRNA) of the parasite (Verweij et al., 2003).

For assessing the molecular diversity of the parasite, we adopted a sequence-based multilocus genotyping (MLST) scheme targeting the genes encoding for the *ssu* rRNA, the glutamate dehydrogenase (*gdh*), β-giardin (*bg*) and triose phosphate isomerase (*tpi*) proteins of the parasite. For assessing the molecular diversity of *G. duodenalis* at the assemblage level, a nested PCR was used to amplify a fragment of the *ssu* rRNA gene (Appelbee et al., 2003; Hopkins et al., 1997). The molecular diversity of the parasite at the sub-assemblage level was investigated only in *Giardia* isolates that tested positive by qPCR and yielded cycle threshold (C_T) values $\leq$32. A semi-nested PCR was used to amplify a fragment of the *gdh* gene (Read et al., 2004), and nested PCRs were used to amplify fragments of the *bg* and *tpi* genes, respectively (Cacciò et al., 2002; Lalle et al., 2005; Sulaiman et al., 2003).

2.7 | Molecular detection and characterisation of *Cryptosporidium* sp

The presence of *Cryptosporidium* sp. was assessed using a nested-PCR protocol to amplify a fragment of the *ssu* rRNA gene of the parasite (Tiangtip & Jongwutiwes, 2002). Subtyping tools based on the amplification of partial sequences of the 60-kDa glycoprotein (*gp60*) gene were used to ascertain intra-species genetic diversity in samples that tested positive for *C. canis* (Jiang et al., 2021) and *C. parvum* (Feltus et al., 2006).

2.8 | Molecular detection of *Strongyloides* sp

Identification of *Strongyloides* sp. was carried out by a qualitative qPCR method using genus-specific primers targeting the *ssu* rRNA gene of the parasite (Saugar et al., 2015; Verweij et al., 2009).

2.9 | Molecular detection of *Blastocystis* sp

Identification of *Blastocystis* sp. was achieved by a direct PCR protocol targeting a fragment of the *ssu* rRNA gene of the parasite (Scicluna et al., 2006).

2.10 | Molecular detection and characterisation of *Enterocytozoon bieneusi*

Detection of *E. bieneusi* was conducted by a nested PCR protocol to amplify a fragment of the internal transcribed spacer (ITS) region as well as portions of the flanking large and small subunit of the ribosomal RNA gene as previously described (Buckholt et al., 2002).

2.11 | General PCR and electrophoretic procedures

Detailed information on the PCR cycling conditions and oligonucleotide sequences used for the molecular identification and/or characterisation of the protozoan parasites investigated in the present study is presented in Tables S1 and S2, respectively.

All qPCR protocols described above were carried out on a Corbett Rotor Gene™ 6000 real-time PCR system (Qiagen). Reaction mixes included 2× TaqMan® Gene Expression Master Mix (Applied Biosytems, Foster City, CA, USA) or 1× Quantimix EasyMaster Mix (Biotools B&M Laboratories, Madrid, Spain) and 0.5 μL of 50× Sybr-Green (Invitrogen, San Diego, CA, USA) for detection of *G. duodenalis* and *Strongyloides* sp., respectively. 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 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde, Germany), and 5–10 μL MyTAQ™ Reaction Buffer containing 5 mM dNTPs and 15 mM $MgCl_2$. Laboratory-confirmed positive and negative DNA samples of human and animal origin for each parasitic species investigated were routinely used as controls and included in each round of PCR. PCR amplicons were visualised on 1.5% D5 agarose gels (Conda, Madrid, Spain) stained with Pronasafe (Conda) nucleic acid staining solutions. A 100 bp DNA ladder (Boehringer Mannheim GmbH, Baden-Wurttemberg, Germany) was used for the sizing of obtained amplicons.

2.12 | Sanger sequencing analyses

Positive-PCR products of the expected size were directly sequenced in both directions using appropriate internal primer sets (Table S2). DNA sequencing was conducted by capillary electrophoresis using the BigDye® Terminator chemistry on an on ABI PRISM 3130 automated DNA sequencer (Applied Biosystems). Generated DNA consensus sequences were aligned to appropriate reference sequences obtained in GenBank using the Basic Local Alignment Search Tool (BLAST) and the MEGA X software (Kumar et al., 2018) for species confirmation and genotype identification. The sequences obtained in this study have been deposited in GenBank under accession numbers OQ722809–OQ722811 and OQ679953–OQ679968 (*G. duodenalis*), OQ722812–OQ722814 and OQ679969 (*Cryptosporidium* sp.), and OQ722933 (*Enterocytozoon bieneusi*).

2.13 | Statistical analysis

The chi-square test (χ^2) was used to determine potential statistically significant associations between the occurrence of individual enteric parasite species in dogs and cats and variables including age, sex, seasonality, anti-parasitic treatment in the 3 months previous to sampling, presence of gastrointestinal or respiratory clinical manifestations and faecal consistency (1: hard, 2: formed, 3: soft, 4: liquid). A P-value < 0.05 was considered statistically significant.

The Cohen's Kappa test was estimated to assess the agreement of the diagnostic results obtained with the Stick Crypto-Giardia (Operon) ICT test and conventional microscopy examination. Cohen's Kappa ranges between 0 (no agreement between the two raters) and 1 (perfect agreement between the two raters). A Cohen's kappa value between 0.81 and 0.99 was considered as 'near perfect agreement'.

3 | RESULTS

In this study, we tested faecal samples from 252 dogs and 35 cats that attended a small animal clinic during the period 2017—2021 in Madrid, central Spain (Table S3). The median age of the investigated dogs was 4.5 months [range: 1—156; standard deviation (SD): 42.3]. The median age of the investigated cats was 4.0 months (range: 1—228; SD: 49.1]. Near half of the dogs (49.6%, 125/252) and cats (54.3%, 19/35) were ≤4 months of age. The male/female ratios for dogs and cats were 1.3 in both cases. Collection of canine and feline faecal samples was similarly distributed across seasons (spring: $n = 93$; summer: $n = 37$; autumn: $n = 78$, winter; $n = 79$). Overall, 32.9% (83/252) of dogs and 11.4% (4/35) of cats received anthelmintic treatment in the 3 months previous to sample collection. Gastrointestinal and respiratory clinical manifestations were observed in 51.6% (130/252) and 5.2% (13/252) of dogs and in 28.6% (10/35) and 5.7% (2/35) of cats, respectively. Most of the faecal samples collected were formed both in dogs (55.2%, 139/252) and cats (77.1%, 27/35).

3.1 | Microscopy

Overall, 48.8% (123/252) of dogs and 48.6% (17/35) of cats were infected by at least a single species of enteric parasites. Infections by protists were more frequent than infections by helminth parasites in dogs (43.7% vs. 9.5%), whereas the opposite pattern was observed in cats (14.3% vs. 45.7%).

In dogs, *G. duodenalis* (cysts) was the most common parasite species found (40.9%, 103/252; 95% CI: 34.7—47.2), followed by *Cystoisospora* sp. (7.1%, 18/252; 95% CI: 4.3—11.0), and *T. canis* (5.2%, 13/252; 95% CI: 2.8—8.7). *Cryptosporidium* sp. oocysts, *T. leonine* eggs, *D. caninum* proglottids, hookworms eggs, and *Strongyloides* sp. larvae were all detected at low (<2%) prevalence rates (Table 1).

TABLE 1 Microscopy-based frequencies of enteric parasites in the canine ($n = 252$) and feline ($n = 35$) populations investigated in the region of Madrid (Spain), and 95% confidence intervals (95% CI) are indicated.

		Dogs ($n = 252$)			Cats ($n = 35$)			All ($n = 287$)		
Group	Species	Pos. (n)	%	95% CI	Pos. (n)	%	95% CI	Pos. (n)	%	95% CI
Protozoa	*Giardia duodenalis*	103	40.9	34.7–47.2	5	14.3	4.8–30.3	108	37.6	32.0–43.5
	Cystosisospora sp.	18	7.1	4.3–11.0	1	2.9	0.07–14.9	19	6.6	4.0–10.2
Nematoda	*Toxocara canis/cati*	13	5.2	2.8–8.7	7	20.0	8.4–36.9	20	7.0	4.3–10.6
	Toxascaris leonina	4	1.6	0.4–4.0	1	2.9	0.07–14.9	5	1.7	0.6–4.0
	Fam. Ancylostomatidae	3	1.2	0.3–3.4	0	0.0	–	3	1.1	0.2–3.0
	Strongyloides sp.	2	0.8	0.1–2.8	0	0.0	–	2	0.7	0.08–2.5
Cestoda	*Dipylidium caninum*	4	1.6	0.4–4.0	2	5.7	0.7–19.2	6	2.1	0.8–4.5
	Joyeuxiella sp.	0	0.0	–	7	20.0	8.4–36.9	7	2.4	1.0–5.0

In cats, *Joyeuxiella* sp. and *T. cati* were the dominant enteric parasite species found (20.0%, 7/35; 95% CI: 8.4–36.9 each), followed by *G. duodenalis* (14.3%, 5/35; 95% CI: 4.8–30.3), *D. caninum* (5.7%, 2/35; 95% CI: 0.7–19.2) and *Cystoisospora felis* and *T. leonina* (2.9%, 1/35; 95% CI: 0.07–14.9 each) (Table 1).

Coinfections by two or more enteric parasite species were identified in 10.3% (26/252) of dogs and 14.3% (5/35) of cats, respectively. The combination *G. duodenalis* + *Cystoisospora* sp. was the coinfection most frequently detected in dogs (42.3%, 11/26) and *G. duodenalis* + *T. cati* in cats (40.0%, 2/5). The distribution of infections by single or multiple enteric parasites in the joint canine and feline populations is shown in Table 2.

TABLE 2 Microscopy-based frequencies of enteric parasites in monoinfection and coinfection in the canine ($n = 252$) and feline ($n = 35$) populations investigated in the region of Madrid (Spain).

Parasite species	Positive (n)	Frequency (%)[a]
None	147	51.2
In monoinfection		
G. duodenalis	83	28.9
Toxocara canis/cati	8	2.8
Joyeuxiella sp.	7	2.4
Cystoisospora sp.	5	1.7
Dipylidium caninum	3	1.0
Fam. Ancylostomatidae	3	1.0
Toxascaris leonina	1	0.3
In co-infection		
G. duodenalis + *Cystoisospora* sp.	12	4.2
G. duodenalis + *Toxocara canis/cati*	8	2.8
G. duodenalis + *D. caninum*	2	0.7
T. canis + *Cystoisospora* sp.	2	0.7
T. canis+ *T. leonine*	2	0.7
G. duodenalis + *Strongyloides* sp.	1	0.3
G. duodenalis + *Toxascaris leonina*	1	0.3
T. cati + *D. caninum*	1	0.3
G. duodenalis + *Strongyloides* sp. + *T. leonina*	1	0.3
Total	287	100

[a] Over the total of fecal samples ($n = 287$) examined.

3.2 | Diagnostic performance of immunochromatographic rapid test using microscopy as gold standard

The ICT test yielded positive results for *G. duodenalis* in 113 faecal samples (39.4%, 113/287), 106 from dogs (42.1%; 106/252) and seven from cats (20.0%; 7/35). The frequency of agreement between microscopy and ICT was 98.3%. The ICT test identified five more *Giardia*-positive samples (three in dogs and two in cats) than conventional microscopy. The Kappa coefficient showed an almost perfect agreement between both diagnostic methods ($\kappa = 0.963$).

In addition, five canine faecal samples (2.0%, 5/252) were tested positive for *Cryptosporidium* sp. by ICT. No feline faecal samples were tested positive for this pathogen by ICT.

3.3 | Molecular characterisation of *Giardia duodenalis* isolates

Out of the 113 canine and feline faecal samples with a positive result either by microscopy or ICT, a total of 86 (82 from dogs and four from cats) were available for qPCR testing. Out of the 82 canine isolates tested, 86.6% (71/82) were confirmed by qPCR, yielding cycle threshold (C_T) values ranging from 18.6 to 38.6 (median: 30.8; SD: 4.8). Out of the four feline isolates tested, 50% (2/4) were confirmed by qPCR, yielding C_T values ranging of 34.8 and 41.8, respectively.

A total of 11 *G. duodenalis* isolates of canine origin were successfully amplified at one or more of the four (*ssu* rRNA, *gdh*, *bg* and *tpi*) genetic

TABLE 3 Multilocus sequence typing results of the 11 *G. duodenalis*-positive samples of canine origin successfully genotyped at any of the four loci investigated in the present survey.

Sample ID	Host	Age (months)	C_T value in qPCR	*ssu* rRNA	*gdh*	*bg*	*tpi*	Assigned genotype
166	Dog	18	22.6	–	A1	A1	–	A1
214	Dog	3	20.0	–	C	C+D	C	C+D
217	Dog	12	24.1	–	C	–	–	C
230	Dog	2	18.6	–	D	D	C	C+D
242	Dog	2	28.6	C	–	–	–	C
245	Dog	3	23.8	–	D	D	–	D
250	Dog	2	29.3	–	D	D	–	C
252	Dog	12	33.9	A	–	–	–	A
255	Dog	24	26.4	–	C	–	–	C
258	Dog	7	22.7	D	D	D	–	D
279	Dog	4	26.9	–	D	D	–	D

markers used for genotyping purposes (Table 3). Four isolates were amplified at a single locus or two loci (36.4% each), and the remaining three (27.2%) at three independent loci. None of the 11 isolates were simultaneously amplified at the four loci. Nucleotide sequence analyses revealed the presence of zoonotic assemblage A (18.2%, 2/11) and canine-adapted assemblages C (36.3%, 4/11) and D (27.3%, 3/11). An additional isolate (18.2%, 2/11) was identified as a mixed C+D infection (Table 3).

Table 4 summarises the molecular data generated at the *ssu* rRNA, *gdh*, *bg* and *tpi* loci. The three nucleotide sequences amplified at the *ssu* rRNA gene were assigned to assemblages A, C and D, respectively. These sequences were identical, or differed by a single nucleotide polymorphism (SNP), with their respective reference sequences.

At the *gdh* locus, one isolate was confirmed as sub-assemblage A1, differing by a single SNP from its reference sequence (GenBank accession number L4050917) (Table 4). Out of the three assemblage C sequences identified, two showed 100% identity with the reference U60984, with the remaining one differing from it by a single SNP. A higher level of genetic diversity was observed among the five assemblage D sequences found: only one was identical to reference sequence, whereas the remaining four differed from it by two to four SNPs (Table 5). Out of the six isolates amplified at the *bg* locus, one showed 100% identity with sub-assemblage AI reference sequence AY655702, confirming the results previously obtained at the *ssu* rRNA and *gdh* loci. Four isolates were identified as assemblage D, one being identical to reference sequence AY545647, and the other three differing from it by one to three SNPs. The sixth *bg* isolate corresponded to a mixed C+D infection. Taking AY545647 as reference sequence, this isolate differed from it by 33 SNPs, 32 of them corresponding to ambiguous (double peak) positions (Table 4). The two isolates amplified at the *tpi* loci were assigned to assemblage C, differing by four to six SNPs between them and from reference sequence AY228641 (Table 4).

None of the two feline isolates with a *Giardia*-positive result at microscopy examination or ICT were genotyped at the *ssu* rRNA, *gdh*, *bg* or *tpi* loci.

3.4 | Molecular characterisation of *Cryptosporidium* sp. isolates

All five canine samples that tested positive for *Cryptosporidium* sp. by ICT yielded amplicons of the expected size in *ssu*-PCR. Sequence analyses of the obtained amplicons allowed the identification of zoonotic *C. parvum* (n = 1) and host-specific *C. canis* (n = 4) (Table 4). The *C. parvum* sequence was identified as the bovine genotype of the parasite, differing from reference sequence AF112571 by six SNPs including the distinctive TAAT deletion in positions 686_689 (Table 4). This isolate was not be amplified at the *gp60* locus.

The four *C. canis* sequences showed 100% identity with reference sequence AF112576. Only a single isolate could be molecularly characterised at the *gp60* locus. Sequence analysis confirmed the presence of a novel *C. canis* subtype family that we named XXi1 in agreement with the established nomenclature for *Cryptosporidium* subtype families (Xiao & Feng, 2017).

Figure 1 shows the maximum-likelihood tree generated with representative sequences of the eight *C. canis* subtype families (XXa, XXb, XXc, XXd, XXe, XXf, XXg and XXh) described to date. As expected, our XXi1 isolate formed an independent cluster in the topology of the generated tree.

3.5 | Molecular identification and characterisation of *Strongyloides* sp., *Blastocystis* sp. and *E. bieneusi*

The two canine isolates positive to *Strongyloides* sp. by conventional microscopy examination were confirmed by qPCR. Obtained qPCR C_T values were 28 and 32.

To maximise the generated material, all faecal DNA samples available from canine (n = 82) and feline (n = 4) origin were re-assessed for the presence of the Stramenopile *Blastocystis* sp. and the Microsporidia *E. bieneusi*. None of these two protist species were identified at

TABLE 4 Frequency and molecular diversity of the *G. duodenalis*, *Cryptosporidium* spp. and *E. bieneusi* sequences successfully genotyped in the canine population investigated in the region of Madrid (Spain). GenBank accession numbers are provided.

Species	Genotype	Sub-genotype	Locus	No. isolates	Reference sequence	Stretch	Single nucleotide polymorphisms	GenBank ID
Giardia duodenalis	A	–	*ssu* rRNA	1	M54878	51–289	G113T	OQ722809
	C	–	*ssu* rRNA	1	AF199449	14–290	A199R	OQ722810
	D	–	*ssu* rRNA	1	AF199443	70–291	None	OQ722811
	A	AI	*gdh*	1	L40509	64–491	C447Y	OQ679953
	C	–	*gdh*	2	U60984	76–496	None	OQ679954
	C	–	*gdh*	1	U60984	76–491	A212T	OQ679955
	D	–	*gdh*	1	U60986	67–491	None	OQ679956
	D	–	*gdh*	1	U60986	73–491	T240C, T429C, G441A	OQ679957
	D	–	*gdh*	1	U60986	64–423	T240C, T429C, G441A, T459A	OQ679958
	D	–	*gdh*	1	U60986	125–417	T312Y, C375Y	OQ679959
	D	–	*gdh*	1	U60986	76–496	C375Y, T429Y, G441R	OQ679960
	A	AI	*bg*	1	AY655702	33–523	None	OQ679961
	D	–	*bg*	1	AY545647	98–596	None	OQ679962
	D	–	*bg*	1	AY545647	98–590	A159G, A201G, T251Y	OQ679963
	D	–	*bg*	2	AY545647	102–555	A201G	OQ679964
	D	–	*bg*	1	AY545647	102–590	A201G, C207Y	OQ679965
	C+D	–	*bg*	1	AY545647	96–594	T123Y, T132Y, T150Y, A159W, T165K, T177Y, G183S, A194R, A201G, A202R, C207S, A231M, T243Y, T255Y, G273R, T276Y, A282M, A291R, C309Y, A312M, G327S, A387R, T390Y, T426Y, T441Y, G496R, T510Y, T513Y, T519Y, A552R, A570R, A573C, T579K	OQ679966
	C	–	*tpi*	1	AY228641	21–532	G37A, C63T, G136T, T316C, C369T, A379C	OQ679967
	C	–	*tpi*	1	AY228641	19–531	C49Y, G136K, T316Y, A368R, C369Y, A379M	OQ679968
Cryptosporidium parvum	–	–	*ssu* rRNA	1	AF112571	528–1030	A646G, T649G, 686_689DelTAAT, T693A, T709C	OQ722812
Cryptosporidium canis	–	–	*ssu* rRNA	4	AF112576	538–1021	None	OQ722813
	–	XXi1[a]	*gp60*	1	–	–	–	OQ679969
Enterocytozoon bieneusi	–	PtEb IX	ITS	1	AF059610	34–416	None	OQ722933

bg, *β*-giardin; Del, deletion; *gdh*, glutamate dehydrogenase; ITS, internal transcribed spacer; K, T/G; M, C/A; R, A/G; S, G/C; *ssu* rRNA, small subunit ribosomal RNA; *tpi*, triose phosphate isomerase; Y: C/T.

microscopy examination. All analysed samples were tested negative for *Blastocystis* sp., but a dog isolate were tested positive for *E. bieneusi*. Nucleotide sequence analysis revealed the presence of canine-adapted genotype PtEb IX. This sequence showed 100% identity with reference sequence AF059610 (Table 4).

3.6 | Risk association analysis

Table 5 summarises the results of the statistical analyses conducted to demonstrate potential associations between individual enteric parasites and the epidemiological variables considered in the study. To

TABLE 5 Frequencies of enteric parasites in the joint canine ($n = 252$) and feline ($n = 35$) populations investigated in the region of Madrid (Spain) according to age group, sex, seasonality, clinical manifestations and faecal consistency. Bolded values indicate statistical significance.

Variable	Animals (*n*)	Any parasite (%)	*Cystoisospora* sp. (%)	*Giardia duodenalis* (%)	*Toxocara canis* (%)	*Toxascaris leonina* (%)	Fam. Ancylostomatidae (%)	*Strongyloides* sp. (%)	*Dipylidum caninum* (%)	*Joyeuxiella* spp. (%)
Age (months)										
≤ 4	146	**98 (67.1)**	**16 (11.0)**	**76 (52.1)**	**18 (12.3)**	4 (2.7)	1 (0.7)	2 (1.4)	5 (3.4)	1 (0.7)
>4 ≤ 8	22	11 (50.0)	2 (9.1)	9 (40.9)	1 (4.5)	0 (0.0)	1 (4.5)	0 (0.0)	0 (0.0)	0 (0.0)
>8 ≤ 12	18	10 (55.6)	0 (0.0)	9 (50.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
> 12 ≤ 24	24	10 (41.7)	0 (0.0)	7 (29.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	**3 (12.5)**
> 24	77	**11 (14.3)**	**1 (1.3)**	**7 (9.1)**	**1 (1.3)**	1 (1.3)	0 (0.0)	0 (0.0)	1 (1.3)	3 (3.9)
Total	287	140 (48.8)	19 (6.6)	108 (37.6)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		***P* < 0.001**	***P* = 0.025**	***P* < 0.001**	***P* = 0.009**	*P* = 0.723	*P* = 0.116	*P* = 0.746	*P* = 0.579	***P* = 0.008**
Sex										
Male	164	84 (51.2)	10 (6.1)	65 (39.6)	13 (7.9)	5 (3.0)	2 (1.2)	1 (0.6)	4 (2.4)	4 (2.4)
Female	123	56 (45.5)	9 (7.3)	43 (35.0)	7 (5.7)	0 (0.0)	1 (0.8)	1 (0.8)	2 (1.6)	3 (2.4)
Total	287	140 (48.8)	19 (6.6)	108 (37.6)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		*P* = 0.340	*P* = 0.681	*P* = 0.419	*P* = 0.462	*P* = 0.051	*P* = 0.738	*P* = 0.838	*P* = 0.634	*P* = 1.000
Seasonality										
Spring	93	**34 (36.6)**	6 (6.5)	**27 (29)**	6 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.2)	1 (1.1)
Summer	37	14 (37.8)	2 (5.4)	11 (29.7)	3 (8.1)	1 (2.7)	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)
Autumn	78	40 (51.3)	4 (5.1)	29 (37.2)	7 (9.0)	3 (3.8)	**3 (3.8)**	1 (1.3)	4 (5.1)	0 (0.0)
Winter	79	**52 (65.8)**	7 (8.9)	**41 (51.9)**	4 (5.1)	1 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)	**6 (7.6)**
Total	287	140 (48.8)	19 (6.6)	108 (37.6)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		***P* < 0.001**	*P* = 0.798	***P* = 0.013**	*P* = 0.792	*P* = 0.265	***P* = 0.044**	*P* = 0.290	*P* = 0.112	***P* = 0.006**
Deworming <3 months										
Yes	87	48 (55.2)	9 (10.3)	43 (49.4)	**1 (1.1)**	2 (2.3)	**0 (0.0)**	–	2 (2.3)	–
No	31	18 (58.1)	4 (12.9)	10 (32.3)	**7 (22.6)**	0 (0.0)	**2 (6.5)**	–	2 (6.5)	–
Total	118	66 (55.9)	13 (11.0)	53 (44.9)	8 (6.8)	2 (1.7)	2 (1.7)	–	4 (3.4)	–
P-value		*P* = 0.781	*P* = 0.696	*P* = 0.099	***P* < 0.001**	*P* = 0.395	***P* = 0.017**	–	*P* = 0.273	–

(Continues)

TABLE 5 (Continued)

Variable	Animals (*n*)	Any parasite (%)	*Cystoisospora* sp. (%)	*Giardia duodenalis* (%)	*Toxocara canis* (%)	*Toxascaris leonina* (%)	Fam. Ancylostomatidae (%)	*Strongyloides* sp. (%)	*Dipylidum caninum* (%)	*Joyeuxiella* spp. (%)
Digestive signs										
Yes	141	**82 (58.2)**	**15 (10.6)**	**70 (49.6)**	9 (6.4)	2 (1.4)	2 (1.4)	2 (1.4)	2 (1.4)	1 (0.7)
No	146	**58 (39.7)**	**4 (2.7)**	**38 (26.0)**	11 (7.5)	3 (2.1)	1 (0.7)	0 (0.0)	4 (2.7)	6 (4.1)
Total	287	140 (48.8)	19 (6.6)	108 (37.6)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		**$P = 0.002$**	**$P = 0.007$**	**$P < 0.001$**	$P = 0.702$	$P = 0.680$	$P = 0.541$	$P = 0.149$	$P = 0.434$	$P = 0.062$
Respiratory signs										
Yes	15	10 (66.7)	0 (0.0)	**9 (60.0)**	2 (13.3)	1 (6.7)	0 (0.0)	**2 (13.3)**	0 (0.0)	0 (0.0)
No	271	130 (48.3)	19 (7.1)	**97 (36.1)**	18 (6.7)	4 (1.5)	3 (1.1)	**0 (0.0)**	6 (2.2)	7 (2.6)
Total	286	140 (49.0)	19 (6.6)	108 (37.8)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		$P = 0.124$	$P = 0.526$	**$P = 0.034$**	$P = 0.573$	$P = 0.324$	$P = 0.909$	**$P < 0.001$**	$P = 0.824$	$P = 0.797$
Faecal consistency										
Hard	1	1 (100)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Formed	166	**67 (40.4)**	**4 (2.4)**	**45 (27.1)**	13 (7.8)	4 (2.4)	1 (0.6)	0 (0.0)	4 (2.4)	6 (3.6)
Soft	90	**56 (62.2)**	**10 (11.1)**	**48 (53.3)**	5 (5.6)	1 (1.1)	1 (1.1)	2 (2.2)	2 (2.2)	1 (1.1)
Liquid	30	16 (53.3)	**5 (16.7)**	14 (46.7)	2 (6.7)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)
Total	287	140 (48.8)	19 (6.6)	108 (37.6)	20 (7.0)	5 (1.7)	3 (1.0)	2 (0.7)	6 (2.1)	7 (2.4)
P-value		**$P = 0.006$**	**$P = 0.005$**	**$P < 0.001$**	$P = 0.908$	$P = 0.755$	$P = 0.605$	$P = 0.221$	$P = 0.861$	$P = 0.493$

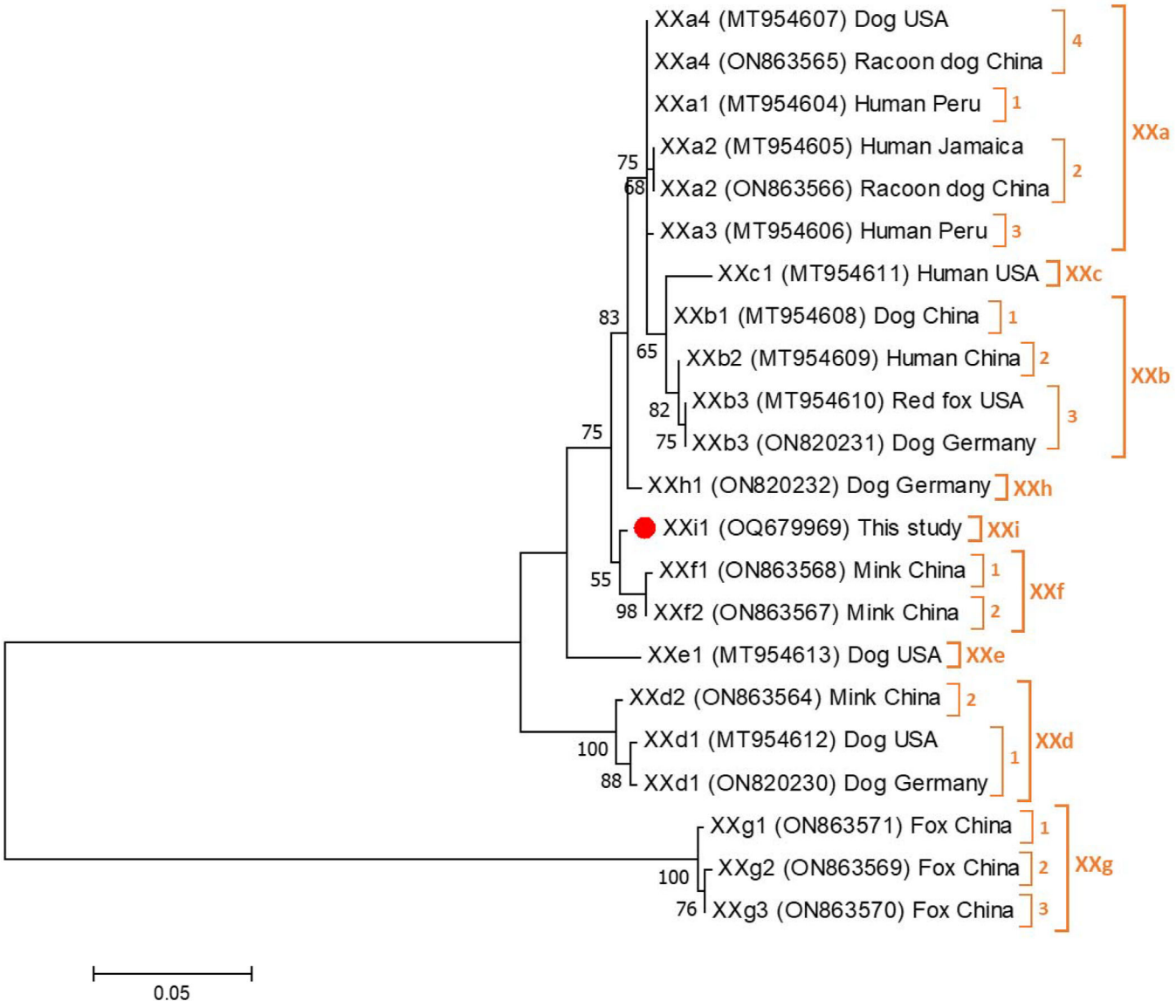

FIGURE 1 Phylogenetic relationship among nine *Cryptosporidium canis* subtype families (XXa–XXh) revealed by a maximum likelihood analysis of the partial *gp60* gene. Substitution rates were calculated by using the general time reversible model. Numbers on branches are percent bootstrapping values over 50% using 1000 replicates. The filled red circle indicates the nucleotide sequence of the novel subtype XXi1 generated in the present study.

increase statistical power canine and feline populations were analysed in combination.

Dogs and cats younger than 4 months of age were at higher risk of infection by any enteric parasite ($P < 0.001$) including *Cystoisospora* sp. ($P = 0.025$), *G. duodenalis* ($P < 0.001$) and *Toxocara* sp. ($P = 0.009$). *Joyeuxiella* sp. was more likely to infect cats in the age group of 12—24 months ($P = 0.008$). Sex was not identified as a risk factor for infections by enteric parasites.

Infections by enteric parasites were significantly higher in the winter months ($P < 0.001$), this being particularly true for *G. duodenalis* ($P = 0.013$). Hookworms infections were only detected during the autumn months ($P = 0.044$).

Dogs and cats receiving antiparasitic treatment the 3 months before sampling were significantly less infected by *Toxocara* sp. ($P < 0.001$) and hookworms ($P = 0.017$) than their untreated counterparts.

Dogs and cats infected by any enteric parasite were more likely to develop gastrointestinal manifestations ($P = 0.002$), being *Cystoisospora* sp. ($P = 0.007$) and *G. duodenalis* ($P < 0.001$) the major contributors to the occurrence of symptoms. Dogs and cats infected with *G. duodenalis* ($P = 0.034$) and *Strongyloides* sp. ($P < 0.001$) were significantly more prone to develop respiratory symptoms.

Soft faecal samples were significantly associated with infections by any enteric parasite ($P = 0.006$), particularly *Cystoisospora* sp. ($P = 0.005$) and *G. duodenalis* ($P < 0.001$).

4 | DISCUSSION

Spain is the fifth largest pet market in Europe, behind the United Kingdom, France, Germany and Italy with 28 million registered pets, among them 6.7 million dogs and 3.8 million cats (International Trade Administration). Four out of 10 Spanish households have pets (ANFAAC, 2021). Given these large number of pet dogs and cats and the proximity and bond of these animals with their owners, understanding and preventing the zoonotic diseases that these companions bring with them are of paramount importance (Baneth et al., 2016; Esch & Petersen, 2013; Overgaauw et al., 2020). Under this approach, we present here novel data on the occurrence, risk factors and genetic variability of intestinal parasites in a large veterinary-visiting pet population in Spain, because few studies have been conducted in animals attending clinical settings in this country (Causapé et al., 1996).

Using microscopy as screening method, we detected *G. duodenalis* cysts in 40.9% and 14.3% canine and feline faecal samples, respectively. Most of these infections occurred in pups and kittens with clinical manifestations, indicating that young animals are more vulnerable to this pathogen. Our results agree with previous studies showing that younger animals are prone to infections with intestinal parasites including *G. duodenalis*, *Cystoisospora* sp. and *Toxocara* sp. that mainly affect animals under 1 year of age (Barutzki & Schaper, 2013) and are more prevalent in breeding kennels than among household animals (Gothe & Reichler, 1990). Lower microscopy-based prevalence rates of 1–16% have been previously reported in all-age dogs from different populations and geographical areas in Spain (Causapé et al., 1996; Dado et al., 2012b; Marbella et al., 2022; Martínez-Carrasco et al., 2007; Martínez-Moreno et al., 2007; Miró et al., 2007; Regidor-Cerrillo et al., 2020). *Giardia duodenalis* infection rates of 4.2% and 5% were found in sheltered and free-roaming cats, respectively, in central Spain (Dado et al., 2012b; Montoya et al., 2018). Using more sensitive detection methods, a composite prevalence rate of 35.4% was obtained by direct immunofluorescence assay (DFA) in different dog populations in eastern Spain (Sanchez-Thevenet et al., 2019), whereas prevalence rates of 29–33 and 6–9% have been reported by PCR in sheltered or owned dogs and cats in the northern part of the country (de Lucio et al., 2017; Gil et al., 2017).

In relation to clinical manifestations, respiratory signs are common in puppies and kittens infected with heavy burdens of migrating larvae of STHs including *Strongyloides stercoralis* and *Toxocara* sp. (Díez Baños et al., 2001; Schnyder et al., 2022). In the present study, respiratory signs have also been associated with *G. duodenalis* infections.

This study showed that ICT performed equally well than conventional microscopy for the detection of *G. duodenalis* cysts in faecal samples (Kappa coefficient, $\kappa = 0.963$). Because ICT is relatively cheap, easy to use and provides diagnostic results in minutes, this assay is increasingly used in veterinary clinical practice to minimise the high amount of labour work required in microscopy testing. Using ICT, *G. duodenalis* has been identified in 25% of faecal samples from dogs and 5–15% from cats (Epe et al., 2010; Montoya et al., 2018) in previous Spanish surveys. Of note, 13 faecal samples that tested positive for *G. duodenalis* by microscopy and/or ICT yielded a negative result by qPCR. Several reasons can explain this discrepancy, including inefficient removal of PCR inhibitors during the DNA extraction and purification process or suboptimal amount of quality template DNA.

Giardia duodenalis is the only *Giardia* species known to naturally infect dogs and cats. It comprises eight distinct genotypes or assemblages (A-H) with marked differences in host specificity and range (Ryan et al., 2021). Assemblages A and B have the broadest host range and are therefore regarded as zoonotic (Cai et al., 2021). Assemblages C and D are mainly found in canine animals, and assemblage E in wild and domestic ungulates, whereas assemblages F, G and H have been mostly reported in felids, rodents and seals, respectively (Ryan et al., 2021). Sporadic reports of zoonotic assemblages A and B in dogs and cats have raised concerns about the actual role of these companion animals as sources of human giardiasis (Ballweber et al., 2010). Despite the fact that studies investigating simultaneously the presence and genetic variants on the parasite in human and animal populations sharing household have not demonstrated the occurrence of zoonotic transmission events (de Lucio et al., 2017; Lucio-Forster et al., 2010; Rehbein et al., 2019), this possibility cannot be overlooked. Indeed, previous molecular-based studies conducted in Spain have found zoonotic assemblages A and B at similar or higher proportions than host-adapted assemblages C, D and F in unowned dogs and cats in northern (Gil et al., 2017) and eastern (Adell-Aledón et al., 2018) Spain. Whether this unexpected high proportion of assemblages A and B in dogs and cats is the result of natural spreading or infections of anthropic nature (e.g. via contamination of water with human faecal material) is something that should be addressed and elucidated in further investigations. In the present study, only 15.5% (11/71) of the *Giardia*-positive samples by qPCR were successfully genotyped at any of the four (*ssu* rRNA, *gdh*, *bg* and *tpi*) loci used for subtyping purposes. This is most likely due to the fact that the *gdh*, *bg* and *tpi* markers are single-copy genes with limited diagnostic sensitivities, making them unsuitable for amplifying samples with little amount of parasitic DNA. This result was expected taking into consideration that most (62%, 44/71) of the *Giardia*-positive samples tested yielded CT values ≥30 at qPCR.

We detected the presence of *Cryptosporidium* sp. antigens by ICT in 2.0% (5/252) of dogs, but none of the 35 cats investigated tested positive by this method. Since apicomplexan (including *Cryptosporidium* sp.) parasites were not specifically searched at microscopy examination, no diagnostic performance agreement between this method and ICT was conducted. Previous studies carried out in Spain have estimated the prevalence of the parasite in dogs at 7.4% by conventional microscopy (Causapé et al., 1996) and at 6.8% by direct immunofluorescence (Sanchez-Thevenet et al., 2019). These surveys did not assess the occurrence of *Cryptosporidium* sp. in feline populations. Using PCR, canine and feline cryptosporidiosis have been estimated in the range of 4–9% (de Lucio et al., 2017; Gil et al., 2017).

The genus *Cryptosporidium* comprises at least 44 recognised species and more than 120 genotypes of uncertain taxonomic status. Nineteen species (mainly *C. hominis*, *C. parvum* and *C. meleagridis*, and, to a lesser extent, *C. canis* and *C. felis*) have been reported in humans (Ryan et al., 2021). Molecular data on the genetic diversity of *Cryptosporidium*

infections in Spanish canine and feline populations are scarce. Host-adapted *C. canis* and *C. felis* were the species primarily found in dogs and cats, respectively (de Lucio et al., 2017; Gil et al., 2017). However, a sheltered dog has been identified carrying *C. hominis* (Gil et al., 2017), a species formerly thought to be human-specific (Widmer et al., 2020). None of the *Cryptosporidium* isolates of canine or feline origin identified in those studies could be subtyped at the *gp60* locus. This trend was also observed in our study, where *C. canis* was predominantly found. Using a specific *gp60* genotyping tool (Jiang et al., 2021), we managed to amplify one of the four *C. canis* isolates and identify it as XXi1, the first member of novel subtype family XXi. To date, nine (XXa to XXh) subtype families of *C. canis* have been recognised in a variety of animal hosts including dogs, foxes, minks and racoon dogs, in addition to humans (Elmahallawy et al., 2023; Jiang et al., 2021; Murnik et al., 2022; Wang et al., 2022). The fifth canine isolate was identified as the bovine genotype of *C. parvum* (unknown subtype family), a zoonotic genetic variant whose primary host species are cattle and humans (Guo et al., 2022). Taken together, these findings suggest that dogs can carry *Cryptosporidium* species (*C. parvum* and *C. canis*) that might represent a public health concern for vulnerable populations such as children and immunocompromised individuals.

The Stramenopile *Blastocystis* sp. was not identified by PCR in any of the 106 canine and seven feline DNA samples available for molecular testing, corroborating previous data about the rare occurrence of *Blastocystis* sp. in various carnivore species (Calero-Bernal et al., 2020; Paulos et al., 2018). However, the Microsporidia *E. bieneusi* was found in a single canine isolate. Nucleotide sequence analysis confirmed the presence of canine-adapted genotype PtEb IX, a genetic variant that has not been described in humans and therefore considered of limited or no zoonotic potential (Li et al., 2019). It should be highlighted that the potential role of pet dogs and cats as sources of human microsporidiosis by *E. bieneusi* should not be overlooked, as both host species have been shown to carry zoonotic genotypes (e.g. BEB6, D and Peru11) of the protist in Spain (Dashti et al., 2019).

The apicomplexan *Cystoisospora* sp. (formerly known as *Isospora* sp.) was identified by conventional microscopy in 7.1% of dogs. This figure is well in the range of those (1–10%) typically found in other Spanish canine populations (Causapé et al., 1996; Gracenea et al., 2009; Martínez-Carrasco et al., 2007; Martínez-Moreno et al., 2007; Miró et al., 2007). Of the four *Cystoisospora* species known to infect dogs (*C. canis*, *C. ohioensis*, *C. burrowsi* and *C. neorivolta*), *C. canis* is morphologically distinctive because of its large-sized oocysts, whereas the latter three are grouped as *C. ohioensis*-like because their oocysts overlap in size (Dubey & Lindsay, 2019). We found oocysts of *Cystoisospora* sp. in 2.9% of feline faecal samples, similar to previous studies in free-rooming cats (2.5%) in central Spain (Montoya et al., 2018). Of note, cats are infected by two *Cystoisospora* species, namely *C. felis* and *C. rivolta* (Dubey, 2018). *Cystoisospora felis* (9.3%) and *Cystoisospora rivolta* (1.7%) have been previously identified in feral cats in Canary Island, Spain (Marbella et al., 2022). Because dogs and cats harbour canine- and feline-adapted *Cystoisospora* species, their role as potential source of human infections (primarily infected by *C. belli*) is considered negligible.

Dogs and cats are known to be suitable hosts for a range of zoonotic STHs of public veterinary health relevance, including members of the genera *Ancylostoma*, *Strongyloides* and *Toxocara* (Gorgani-Firouzjaee et al., 2022; Ketzis & Lucio-Forster, 2020; Traub et al., 2021). Among them, *Toxocara* infections were predominant both in our canine (5.2%) and feline (20.0%) populations. These figures were in agreement with those (dogs: 6–33%; cats: 11–35%) documented in previous epidemiological studies conducted in Spain (Conde Garcia et al., 1989; Marbella et al., 2022; Martínez-Carrasco et al., 2007; Martínez-Moreno et al., 2007; Millán and Casanova, 2009; Miró et al., 2004, 2007; Montoya et al., 2018; Regidor-Cerrillo et al., 2020; Rodríguez-Ponce et al., 2016; Sánchez-Thevenet et al., 2019). On the other hand, infections by *T. leonina* were less common (<3%), and, in the case of hookworms, rare in dogs and absent in cats. Importantly, *Toxocara*-infected puppies or kittens shed large number of eggs with their faeces that contaminate the environment where they undergo development for 2–4 weeks to reach the infective stage (embryonated eggs). Human infections are predominantly acquired from ingestion of embryonated eggs by geophagia in sandpits, parks or playgrounds where cats and dogs have defecated (Dado et al., 2012a; Köchle et al., 2022). Taken together, these findings highlight that (i) our canine and feline populations harboured STH infections (mainly by *T. canis* and *T. cati*) that represent a public health concern, (ii) hygiene practices and regular deworming are crucial to reducing the burden of STH infections and their zoonotic impact and (iii) pet owners must be educated raising the awareness on the relevance of proper waste disposal to prevent environmental contamination. In this regard, it should be noted that STH eggs can rapidly develop into infective forms (e.g. larva 3 in hookworms) under adequate humidity and temperature levels. Precipitations contribute to improve soil moisture conditions, allowing higher survival rates of these infective forms, which, in the case of hookworms, are highly sensitive to desiccation. Rainfall can also extract *Giardia* cysts and *Cryptosporidium* oocysts from soil and grass. Taken together, frequent precipitation events during autumn and winter could potentially explain the superior infection rates by *G. duodenalis* observed during these months in our surveyed pet population (Short et al., 2017; Weaver et al., 2010).

Strongyloides sp. (presumably *S. stercoralis*) was identified in two dogs (0.8%) by microscopy. These infections were confirmed by qPCR, but lack of sequencing data precluded us to unambiguously confirm the species involved. Similar low prevalence rates have been reported in other Spanish canine populations (Sánchez-Thevenet et al., 2019). *Strongyloides* sp. was not identified in any of the faeces of feline origin. However, cats are primarily infected by feline-adapted *Strongyloides* species including *S. felis*, *S. tumefaciens* and *S. planiceps* (Thamsborg et al., 2017), suggesting that they play a negligible role as a source of human strongyloidiasis.

Regarding cestode infections, *D. caninum* was identified in 1.6% of dogs and 5.7% of cats. These rates were similar to those reported in other Spanish epidemiological studies in canine (1–13%) and feline (1–65%) populations (Calvete et al., 1998; Martínez-Carrasco et al., 2007; Martínez-Moreno et al., 2007; Miró et al., 2004, 2007). No members of the family Taeniidae (including *Echinococcus granulosus*) were

identified. Finally, *Joyeuxiella* sp. was the most prevalent enteric parasite (20%, together with *T. cati*) found in cats. Of note, *J. pasqualei* has been identified 55% of stray cats in north-east Spain (Calvete et al., 1998) and in 76% of feral cats in Majorca Island, Spain (Millán and Casanova, 2009).

This study has some limitations that must be taken into consideration when interpreting the results obtained and the conclusions reached. Firstly, conventional microscopy was used as a primary screening method. Because this technique has limited diagnostic sensitivity, it is likely that some of our prevalence data are underestimated. We attempted to minimise this drawback by analysing three consecutive samples from each investigated dog/cat. Secondly, and due to practical reasons, we did not conduct specific staining methods for apicomplexan parasites. This fact precluded us to comparatively assess the diagnostic performance of microscopy versus ICT for the detection of *Cryptosporidium* sp. Thirdly, we only managed to generate genotyping data for a relatively modest number of *G. duodenalis* and *Cryptosporidium* sp. isolates. It is likely that rare or underrepresented genetic variants of these pathogens have been missed. And fourth, obtained results were restricted to relatively small canine and feline population from northern Madrid and might not be representative of the whole epidemiological scenario in this and other regions of the country.

5 | CONCLUSIONS

We provided a thorough account of the occurrence, risk factors and molecular diversity (for *G. duodenalis*, *Cryptosporidium* sp. and *E. bieneusi*) of enteric parasites in well-cared pet dogs and cats in a wealthy area in central Spain. Our results show that one in two dogs and cats was infected by at least one species of protozoan, nematode or cestode parasites. Pups and kittens were at higher risk of infection. Using molecular (PCR and Sanger sequencing) tools, we found that dogs and cats were primarily infected by host-adapted genetic variants of diarrhoea-causing enteric protozoa, but they were also capable of carrying zoonotic strains including *G. duodenalis* assemblage A and *C. parvum*. We described a novel *C. canis* subtype family named XXi and provided diagnostic evidence demonstrating that ICT performed equally well than conventional microscopy for the detection of *G. duodenalis*, representing a cost-effective option for the rapid detection of this pathogen. Dogs and cats were also frequently infected by STH of public health relevance including *T. canis*, *T. cati*, hookworms and, to a much lesser extent, *Strongyloides* sp. Taken together these results indicate that (i) adequate hygiene practices and routine veterinary care are essential to prevent enteric parasite (particularly helminthic) infections and minimise the risk of zoonotic transmission, (ii) vulnerable population (children, the elderly, immunocompromised individuals) should avoid contact with pups/kittens with diarrhoea, (iii) untreated, infected dogs might be major contributors to the environmental contamination with STH eggs and (iv) veterinarian have the duty to notify pet owners about parasitological findings and educate them on their zoonotic potential and public veterinary health implications. Adequate canine and feline waste disposal is important to reduce the spreading of these infections.

AUTHOR CONTRIBUTIONS

Marta Mateo: Conceptualisation; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualisation; Writing—original draft; Writing—review & editing. Ana Montoya: Data curation; Formal analysis; Writing—original draft; Writing—review & editing. Begoña Bailo: Methodology. Alejandro Dashti: Data curation; Investigation; Methodology; Writing—review & editing. Carolina Hernández-Castro: Investigation; Methodology; Writing—review & editing. Pablo Matas: Investigation; Methodology; Writing—review & editing. Lihua Xiao: Data curation; Writing—review & editing. David Carmena: Formal analysis; Investigation; Methodology; Supervision; Validation; Writing—original draft; Writing—review & editing

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

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DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page have been adhered to. No ethical approval was required as this study did not cause any pain or discomfort to the animals because they were not directly or indirectly handled.

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