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Enhancing diagnostic accuracy: Direct immunofluorescence assay as the gold standard for detecting *Giardia duodenalis* and *Cryptosporidium* spp. in canine and feline fecal samples

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Abstract

The enteric protozoan parasites *Giardia duodenalis* and *Cryptosporidium* spp. are common cause of diarrhea in pet dogs and cats, affecting primarily young animals. This comparative study evaluates the diagnostic performance of conventional and molecular methods for the detection of *G. duodenalis* and *Cryptosporidium* spp. infection in dogs and cats.

The compared diagnostic assays included merthiolate-iodine-formalin (MIF) method, lateral flow immunochromatography rapid test (ICT) and real-time PCR; using direct immunofluorescence assay (DFA) as golden standard. The study included the analysis of 328 fecal samples from different dog ($n=225$) and cat ($n=103$) populations.

According to DFA, the overall prevalence of *G. duodenalis* was 24.4% (80/328, 95% CI: 19.8–29.4), varying from 11.6% (12/103, 95% CI: 6.2–19.5) in cats to 30.2% (68/225, 95% CI: 24.3–36.7) in dogs. The overall prevalence of *Cryptosporidium* spp. was 4.0% (13/328, 95% CI: 2.1–6.7), varying from 2.9% (3/103, 95% CI: 0.6–8.3) in cats to 4.4% (10/225, 95% CI: 2.1–8.0) in dogs. MIF was only used for the detection of *G. duodenalis*, which was identified by this method in 22.7% of dogs and 7.8% of cats, respectively. DFA was the most sensitive technique for detecting *G. duodenalis* in samples from dogs and cats (p-value: < 0.001), followed by real-time PCR. Identification of *Cryptosporidium* infections was most effectively accomplished by the combination of DFA and PCR technique (p-value: < 0.001). In addition, epidemiological (sex, age, origin) and clinical (fecal consistency) variables were collected to assess their potential associations with an increased likelihood of infection by *G. duodenalis* and/or *Cryptosporidium* spp. Breeder dogs were more likely to harbor *G. duodenalis* infection (p-value: 0.004), whereas female cats were significantly more infected with *Cryptosporidium* (p-value: 0.003).

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In conclusion, DFA (alone or in combination with PCR) has been identified as the most accurate and cost-effective method for detecting *G. duodenalis* and *Cryptosporidium* spp. in fecal samples from pet dogs and cats. This highlights their importance in both veterinary and clinical settings for enabling prompt treatment and preventing potential transmission to humans.

Keywords Dog, Cat, DFA, MIF, ICT, PCR, Diagnostic performance, Risk factors

Introduction

The enteric protozoan parasites *Giardia duodenalis* and *Cryptosporidium* spp. are of veterinary concern in small animal clinics as they are common causes of acute and prolonged diarrhea in cats and dogs [1–3]. Because infections by both pathogens primarily affect kittens and puppies, accurate and early diagnosis is important to prompt treatment and improve the health status of infected animals [4]. Some genotypes/species of *G. duodenalis* and *Cryptosporidium* spp. have zoonotic potential [5–7]. Several molecular-based studies have failed to demonstrate that companion cats and dogs are significant sources of human giardiasis and cryptosporidiosis [8, 9], suggesting that the overall risk of zoonotic transmission from cats and dogs to humans is low [10]. However, infected pets can pose an underestimated health risk for young children, the elderly, and immunocompromised individuals [11].

The prevalence of *G. duodenalis* infection can vary greatly depending on the animal population analyzed, the clinical status of the surveyed animals, the geographical region of origin, and, importantly, the diagnostic method used. Reported prevalence rates worldwide were in the range of 10–100% in dogs [12] and 1–20% in cats [13]. In Spain, *G. duodenalis* infection rates have been documented, primarily by microscopy examination, at averages of 16.4–40.9% in owned and sheltered dogs [8, 14–18] and 5.9–9.2% in owned and sheltered cats [14]. Similarly, canine and feline infections by *Cryptosporidium* spp. have been documented worldwide in the range of 0–100% and 0–30% [4, 19] respectively, and in Spain a 7.4–14.8% in dogs [20]. No studies of similar characteristics have been conducted in the feline population. However, prevalence rates can vary. Because of their superior diagnostic sensitivities, epidemiological studies based on enzyme-linked immunosorbent assay (ELISA), direct immunofluorescence assay (DFA) or PCR have yielded prevalence rates 2 to 4-fold higher than those based on conventional microscopy [21]. These wide prevalence variations clearly indicate that choosing the most suitable diagnostic technique should be the results of a careful evaluation of the clinical and epidemiological features of the animal population under study as well as of the resources available.

Detection of *G. duodenalis* and *Cryptosporidium* spp. infection in small clinical animal practice can be challenging by reasons including (i) most small veterinary clinics rely on low-sensitivity microscopy-based methods [22], and (ii) subclinical infections characterized by low and intermittent fecal shedding of (oo)cysts are frequent [14, 23]. Several methods are currently available for the detection of *G. duodenalis* and *Cryptosporidium* infection in cats and dogs. Microscopy-based methods include the examination of wet mount preparations (useful in cases of profuse diarrhea) or of concentrated fecal material (the preferred alternative when (oo)cyst counts are low). The latter include methods such as the Telemann flotation technique using saturated saline or zinc sulfate solutions [24, 25], or the merthiolate-iodine-formalin technique (MIF) which, in combination with Lugol's solution, allows the observation of *G. duodenalis* cysts [26, 27]. Detection of *Cryptosporidium* oocysts by microscopy requires specific procedures such as the Ziehl-Neelsen or Heine staining due to the small size of the oocysts, the possibility of confusing them with yeast [28] and because cats and dogs shed low numbers of oocysts per gram, in contrast with other animal species such as cattle [29]. Among the immunodiagnostic methods, several lateral flow immunochromatography rapid tests (ICT) are commercially available, enabling results in as little as 15–30 min [30–33]. However, the reliability of ICT is often hampered by limited diagnostic sensitivities [34] and undesired high rates of false-positive results [35]. Highly sensitive and specific direct immunofluorescence assays (DFA) allow the detection of (oo)cysts in a cost-effective manner and are used as benchmark technique in many clinical veterinary settings [36–38]. Finally, a wide range of single- and multiplex PCR-based methods are also available for the detection of *G. duodenalis* and *Cryptosporidium* spp. infections. Although highly sensitive, their complexity and elevated cost make them unsuited for routine practice in small veterinary clinics. When coupled with Sanger sequencing, molecular methods allow the identification of species and genotypes; this information being particularly relevant in epidemiological and outbreak investigations [39].

The objective of this study was to evaluate the diagnostic performance of common microscopy-based, immunodiagnostic, and molecular methods for the detection of *G. duodenalis* and *Cryptosporidium* infections in fecal samples from different cat and dog populations using DFA as gold standard. As secondary goal, we investigated whether basic epidemiological and clinical factors

increased the odds of feline and canine infections by these pathogens.

Materials and methods

Study design and sample collection

This is a comparative study specifically designed to evaluate the diagnostic performance of the merthiolate-iodine-formalin (MIF) method, a commercially available lateral flow immunochromatography rapid test (ICT), and molecular (PCR) assays for the detection of feline/canine giardiosis and cryptosporidiosis. A commercially available direct immunofluorescence assay (DFA) was used as gold standard based on recommendations of the published literature [40].

The study was conducted against a panel of prospectively collected fecal samples from dogs ($n=225$) and cats ($n=103$) of different age groups (puppy/kitten: 0–6 months; young: 7–12 months; adult: 1–10 years; senior: >10 years) and origin (collectivities, owned, sheltered) and during the period 2020 to 2021. Collectivities refer to breeder dogs and controlled cat colonies under veterinary supervision. In addition, fecal consistency was estimated using the Bristol Stool Chart with a range varying from one (very dry and hard stools) to seven (practically liquid stools) [41, 42]. Animals under antiparasitic treatment were excluded from the survey. The epidemiological and clinical features of the surveyed feline and canine populations are summarized in Table 1.

Coprological analyses were carried out at the Reference Laboratory for the Diagnosis of Parasitic and Vector-Borne diseases in Domestic and Wild Carnivores (PetParasiteLab) of the Faculty of Veterinary Medicine (Complutense University of Madrid, Spain). Molecular testing was conducted at the Parasitology Reference and Research Laboratory of the National Centre for Microbiology (Health Institute Carlos III) in Majadahonda, Spain.

Table 1 Epidemiological and clinical variables of canine and feline populations included

Variable	Cats ($n=103$)		Dogs ($n=225$)	
	n	%	*n*	%
Sex				
Male	60	58.3	130	57.8
Female	43	41.7	95	42.2
Age group				
Puppy/kitten	17	16.5	55	24.4
Young	24	23.3	30	13.3
Adult	58	56.3	123	54.7
Senior	4	3.9	17	7.6
Origin				
Collectivity	4	3.9	13	5.8
Owned	55	53.4	152	67.5
Sheltered	44	42.7	60	26.7
Fecal consistency[a]				
1–2	15	14.6	6	2.7
3–4	68	66.0	120	53.3
5–7	20	19.4	99	44.0

[a]Fecal consistency according to the Bristol stool chart (see text)

Direct immunofluorescence assay (DFA)

The commercial kit Crypto/Giardia Cel IF (CeLLabs, Brookvale, Australia) was used following the manufacturer´s instructions and examined on a Nikon Eclipse Ci-S fluorescence microscope (Nikon, Tokyo, Japan) at 400× magnification. Structures round to oval in shape of the right size (*Giardia* cysts: 8–12 µm; *Cryptosporidium* oocysts: 4–6 µm) stained bright apple green were considered positive (Fig. 1).

Merthiolate-iodine-formalin (MIF) method

This procedure is particularly suited for the detection of *G. duodenalis* cysts in fecal concentrates [27]. Its main application in routinary diagnosis the possibility of detecting morphological differences between active (Fig. 2) and degenerate cysts (Fig. 3). In this study, staining methods were not utilized for the detection of *Cryptosporidium* oocysts due to their considerable time requirements and relatively low sensitivity and specificity [43]. Briefly, 3–5 g of fecal material were thoroughly resuspended in 20 ml of PBS. The homogenate was then filtered through a sieve mesh with a 250 µm diameter to remove large debris. The filtered suspension was then separated into a 10 ml tube and centrifuged at 1,500 rpm for 10 min. Following centrifugation, the supernatant was carefully removed. Two stock solutions, MIF A (50 ml of distilled water, 40 ml of thimerosal 1:1000, 10 ml of formaldehyde and 5 ml of glycerin) and MIF B (100 ml of distilled water, 10 mg of potassium iodide and 5 mg of iodine crystals) were sequentially added to a 10 ml tube. After vortexing and subsequent incubation for 6–8 h at 4 °C, the samples were microscopically examined at 400× magnification. *Giardia* cysts and/or trophozoites typically appear in a light yellow to brown color.

Lateral flow immunochromatography rapid test (ICT)

The commercially available kit Stick Crypto/Giardia (Operon, Zaragoza, Spain) was used for the simultaneous detection of *G. duodenalis* and *Cryptosporidium* spp. following the manufacturer's instructions. A negative result is indicated when only a single green line (control) appears in the results area. A positive result for *Cryptosporidium* spp./*G. duodenalis* infection is indicated when a blue/red line appears alongside the green control line of the control, in the results area. If no lines are visible or only blue/red line are, the test is invalid. The sensitivity and specificity indicated by the manufacturer are 99.9% for both, taken microscopy as the reference technique.

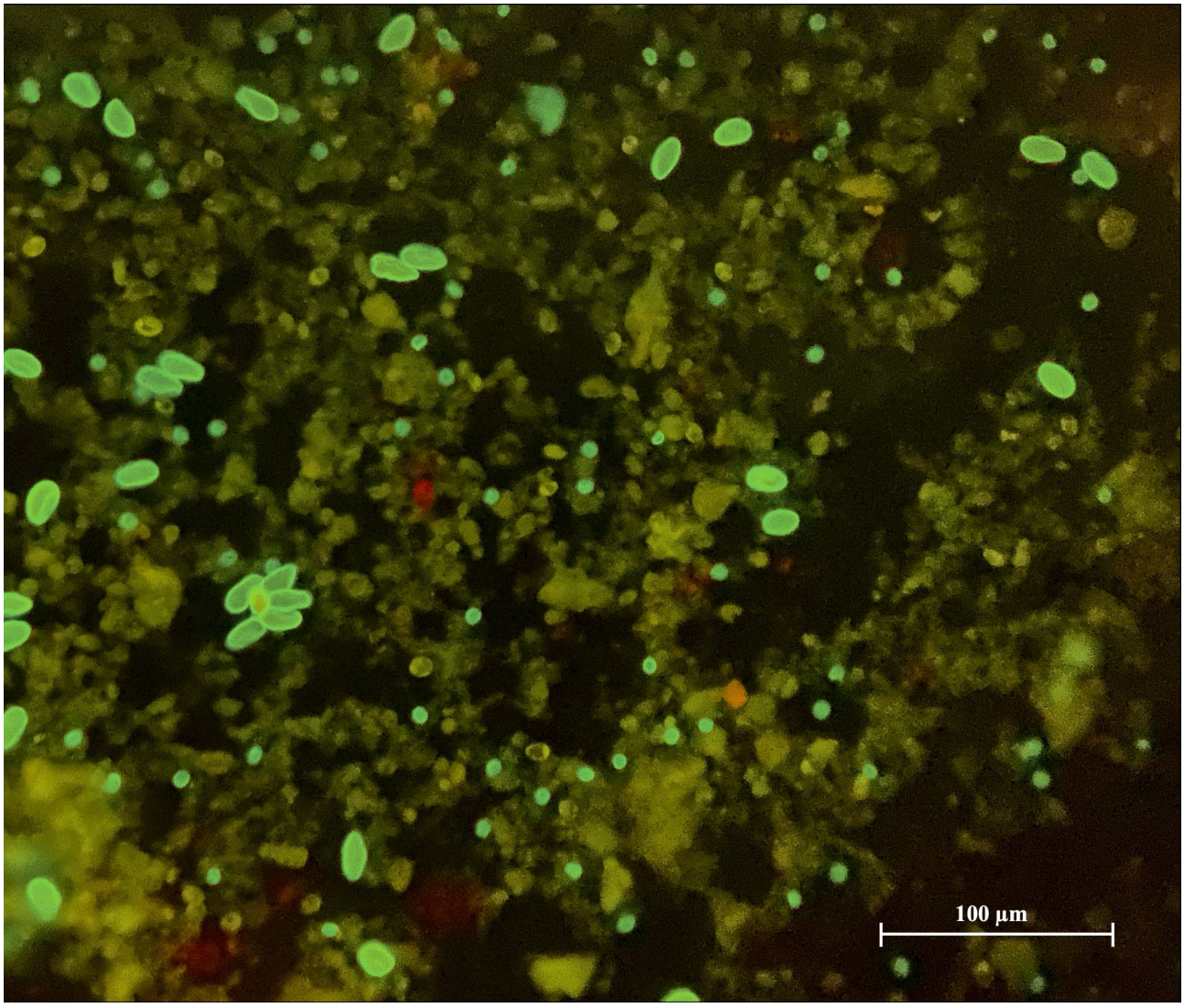

Fig. 1 Fluorescence microscopy image DFA, highlighting *G. duodenalis* cysts (larger size) and *Cryptosporidium* spp. (smaller size). 400x

Fecal DNA extraction and purification

Genomic DNA was isolated from about 200 mg of each concentrated fecal 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 until further PCR analysis.

Real-time PCR for *G. duodenalis* detection

To detect *G. duodenalis* DNA, a real-time PCR protocol was employed to amplify a 62-base pair (bp) segment of the small subunit ribosomal RNA (*ssu*-rRNA) gene of the parasite [44]. Reaction mixtures (25 µl) included 3 µl of template DNA, 12.5 pmol of the primer set Gd-80F (5'–TTGCCAGCGGTGTCCG–3') and Gd-127R (5´–TTGCCAGCGGTGTCCG–3´), 10 pmol of the probe FAM-5'–CCGCGGCGGTCCCTGCTAG–3'-BHQ1, and 1x TaqMan Gene Expression Master Mix (Applied Biosystems, California, EEUU). All PCR runs included both negative and positive controls. Amplification reactions were carried out using a Corbett Rotor-Gene 6000 qPCR cycler (Qiagen). Cycling conditions comprised an initial hold step of 2 min at 55 °C and 15 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 1 min at 60 °C. Samples yielding cycle threshold (C_T) values < 40 was considered positive [45].

PCR for *Cryptosporidium* detection

To detect *Cryptosporidium* spp. DNA, a nested PCR protocol was used to amplify a 587-bp segment of the *ssu*-rRNA gene of the parasite. Reaction mixtures (50 µl) included 3 µl of template DNA, 2.5 units of MyTAQ™ DNA polymerase (Bioline GmbH, Luckenwalde,

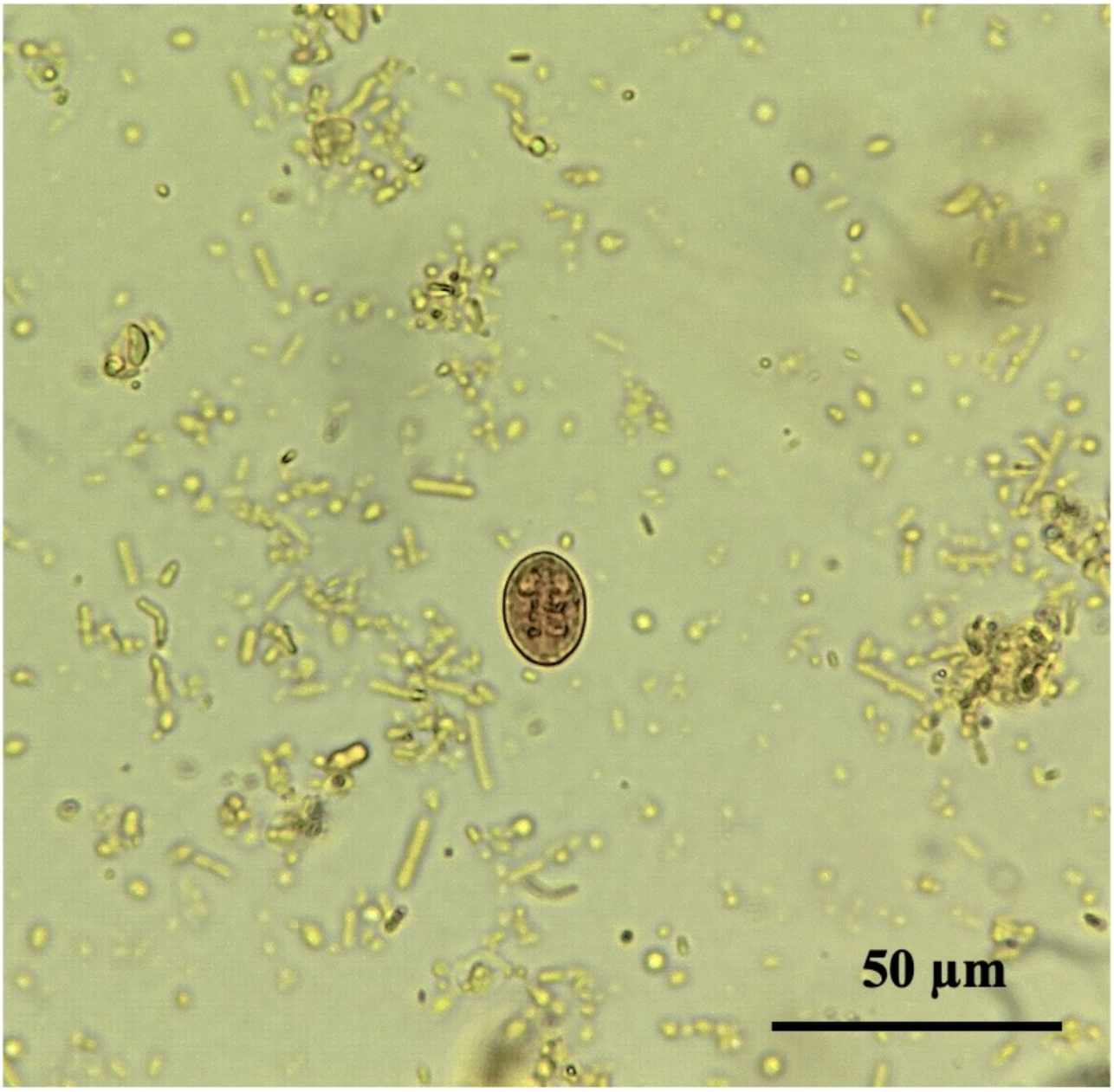

Fig. 2 Faecal smear stained using the MIF technique showing intact *G. duodenalis* cyst. 400x

Germany), and 10 µl of 5x MyTAQ™ Reaction Buffer with 5 mM dNTPs and 15 mM $MgCl_2$. The primer CR-P1 (5'–CAGGGAGGTAGTGACAAGAA–3') and CR-P2 (5'–TCAGCCTTGCGACCATACTC–3') were used in the primary reaction, and the primers CR-P3 (5'-ATTGGAGGGCAAGTCTGGTG-3') and CPB-DIAGR (5'-TAAGGTGCTGAAGGAGTAAGG-3') in the secondary reaction. Both PCR reactions were carried out as follows: one step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 40 s, and 72 °C for 1 min, concluding with a final extension of 72 °C for 10 min. PCR amplicons were visualized on 1.5% D5 agarose gels (Condalab, Madrid, Spain) stained with Pronasafe (Condalab) nucleic acid staining solutions. A 100-bp DNA ladder (Boehringer Mannheim GmbH, Baden-Wurttemberg, Germany) was used to size the obtained amplicons.

Statistical analysis

The chi-squared test was used to study potential associations among the examined variables and the different diagnostic techniques compared. The statistical analyses were performed using the SPSS Statistics package version 17.0 (IBM, Chicago, IL, USA) with a significance level set at $p<0.05$.

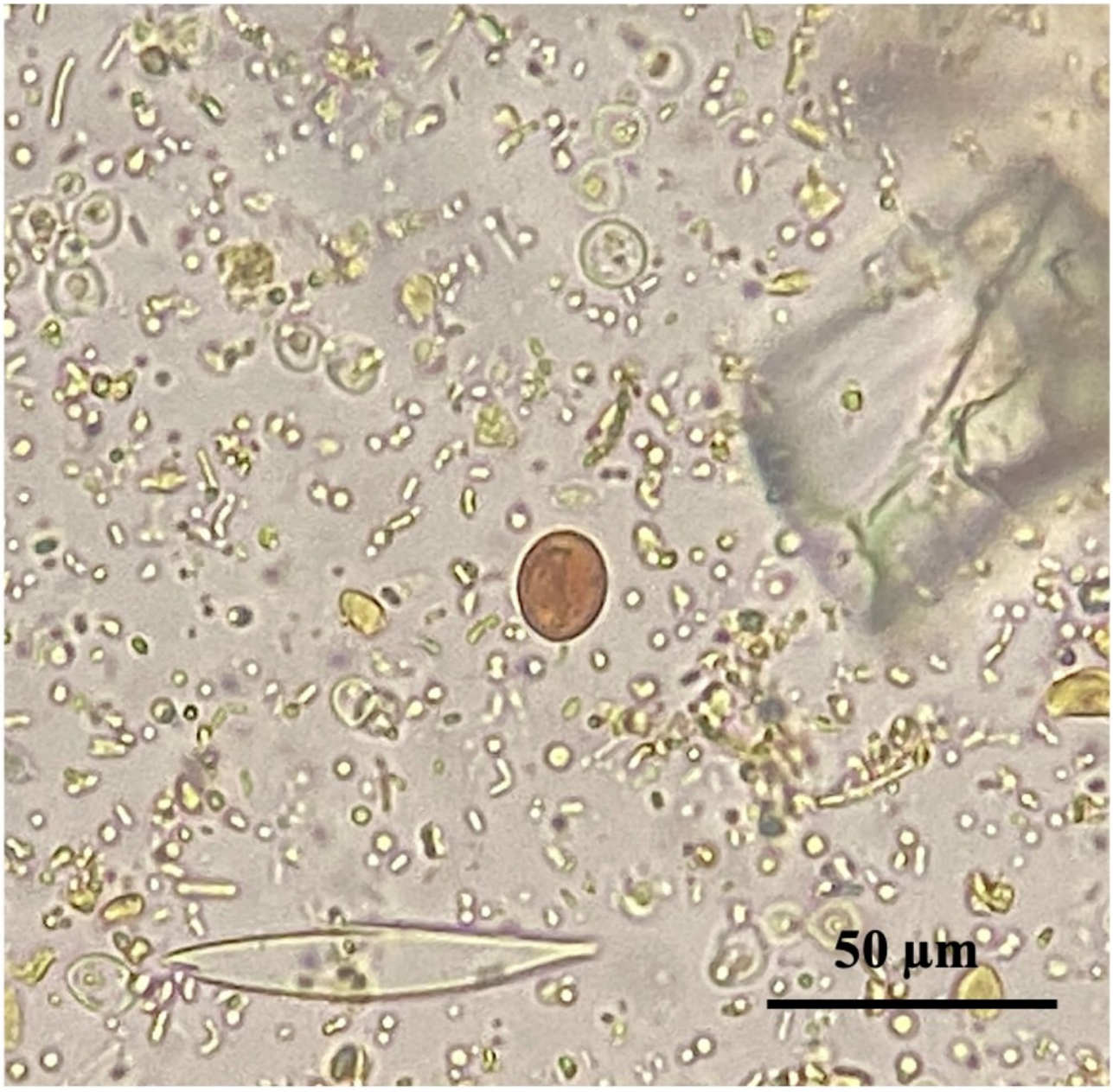

Fig. 3 Faecal smear stained using the MIF technique showing degenerated *G. duodenalis* cyst. 400x

The agreement among the outcomes obtained from the various methods was assessed through the determination of the kappa index. Kappa values ranging from 0.81 to 1 indicated substantial to perfect agreement, showing high consistency between raters. Values from 0.61 to 0.80 indicate substantial agreement, from 0.41 to 0.60 indicate moderate agreement, from 0.21 to 0.40 indicate fair agreement and below 0.20 indicate poor agreement.

Results

A total of 328 fecal samples of feline ($n=103$) and canine ($n=258$) origin were used to assess the diagnostic performance of the detection methods evaluated in the present study. Using DFA as golden standard, the overall prevalence of *G. duodenalis* was 24.4% (80/328, 95% CI: 19.8–29.4), varying from 11.6% (12/103, 95% CI: 6.2–19.5) in cats to 30.2% (68/225, 95% CI: 24.3–36.7) in dogs (Table 2). The overall prevalence of *Cryptosporidium* spp. infection was 4.0% (13/328, 95% CI: 2.1–6.7), varying from 2.9% (3/103, 95% CI: 0.6–8.3) in cats to 4.4% (10/225, 95% CI: 2.1–8.0) in dogs. Coinfection of both

Table 2 Results of *G. duodenalis* and *Cryptosporidium* spp. infection in dogs, cats and total samples by analyzed techniques

	***G. duodenalis* infection**						***Cryptosporidium* spp. infection**					
	Dogs (*n* = 225)		**Cats (*n* = 103)**		**Total (*n* = 328)**		**Dogs (*n* = 225)**		**Cats (*n* = 103)**		**Total (*n* = 328)**	
	Pos. (*n*)	**% (95% CI)**	**Pos. (*n*)**	**% (95% CI)**	**Pos. (*n*)**	**% (95% CI)**	**Pos. (*n*)**	**% (95% CI)**	**Pos. (*n*)**	**% (95% CI)**	**Pos. (*n*)**	**% (95% CI)**
DFA	68	30.2 (24.3–36.7)	12	11.6 (6.2–19.5)	80	24.4 (19.8–29.4)	10	4.4 (2.1–8.0)	3	2.9 (0–6.1)	13	4.0 (2.1–6.7)
MIF	51	22.7 (17.1–28)	8	7.8 (2.5–12.8)	59	18 (13.8–22.1)	ND	ND	ND	ND	ND	ND
ICT	54	24.0 (18.4–29.5	8	7.8 (2.5–12.8)	62	19 (14.7–23.2)	7	3.1 (0.8–5.3)	2	1.9 (0–4.5)	9	2.7 (0.9–4.4)
Real time PCR/PCR	71	31.5 (25.4–37.5)	8	7.8 (2.5–12.8)	79	24 (19.3–28.6)	4	1.7 (0.1–3.3)	3	2.9 (0–6.1)	7	2.1 (1.8–5.9)

ND: not done. DFA: Direct Fluorescence Assay; MIF: Merthiolate-iodine-formalin; ICT: immunochromatography rapid test

parasites was detected in 2.4% (8/328) of samples. Of them, 0.9% (1/103) were of feline origin and 3.1% (7/225) of canine origin.

Table 3 summarizes the diagnostic performance of MIF, ICT, and real-time PCR for the detection of *G. duodenalis* infection in both feline and canine fecal samples (n=328) using DFA as golden standard. The MIF technique achieved diagnostic sensitivity and specificity values of 58.7% and 95.1% for a moderate agreement with DFA (kappa value: 0.59). Both ICT and real-time PCR methods performed better, achieving diagnostic sensitivity and specificity values of 68.7–78.7% and 97.1–93.5% (respectively) for a substantial agreement with DFA (kappa values: 0.71 and 0.72, respectively).

Table 4 summarizes the diagnostic performance of ICT and PCR for the detection of *Cryptosporidium* spp. infection in both feline and canine fecal samples (n=328) using DFA as golden standard. The ICT assay achieved diagnostic sensitivity and specificity values of 46.1% and 99.0% for a moderate agreement with DFA (kappa value: 0.53). The PCR assay achieved diagnostic sensitivity and specificity values of 38.4% and 99.3% for a moderate agreement with DFA (kappa value: 0.48).

Giardia duodenalis was more prevalently found in dogs than in cats regardless the diagnostic method used (p-value: <0.001; Additional Table 2). However, no statistically significant differences in *Cryptosporidium* spp. infection rates were observed between the feline and canine populations, regardless of the diagnostic method used (p-value: 0.5; Additional Table 3).

Table 5 shows the prevalence rates of *G. duodenalis* and *Cryptosporidium* spp. infection (as determined by DFA) in the surveyed canine population according to the epidemiological (sex, age group, origin) and clinical (fecal consistency) variables considered in the study. Dogs living in collectivities were more likely to be infected by *G. duodenalis* (p-value: 0.04). Similarly, female cats were positively associated with a higher odds of *Cryptosporidium* spp. infection than male cats (p-value: 0.03, Table 6).

Discussion

There are few studies comparing four diagnostic techniques for the detection of the diarrhea-causing enteric protozoan parasites *G. duodenalis* and *Cryptosporidium* in naturally infected cats and dogs [46]. Strengths of this survey include (i) the analysis of a relatively large panel of fecal samples from a variety of canine and feline populations reflecting different epidemiological scenarios, and (ii) the inclusion in the comparative performance analysis of the conventional microscopy (MIF), immunodiagnostic (DFA, ICT), and molecular (PCR) methods more often used for diagnostic purposes.

Previous studies have proposed DFA as the gold standard method for routine testing of *G. duodenalis* and

Table 3 Methods performance for detecting *G. duodenalis* infection in feline and canine fecal samples

Diagnostic methods	Result	Positive (*n*)	Negative (*n*)	Kappa	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	DFA% (Positive/*n*)
MIF	Positive	47	12	0.59	58.7 (52.7–63.3)	95.1 (92.7–97.4)	0.79	0.87	17.9 (59/328)
	Negative	33	236						
ICT	Positive	55	7	0.71	68.7 (63.6–73.7)	97.1 (95.2–98.9)	0.88	0.9	18.9 (62/328)
	Negative	25	241						
Real-time PCR	Positive	63	16	0.72	78.7 (74.2–83.1)	93.5 (90.8–96.1)	0.79	0.93	24.0 (79/328)
	Negative	17	232						

n = 328

DFA: Direct Fluorescence Assay; MIF: Merthiolate-iodine-formalin; ICT: immunochromatography rapid test

NPV: Negative predictive value; PPV: Positive predictive value. DFA was used as gold standard. 95% Confidence Intervals (95% CI) are indicated

Table 4 Methods performance for detecting *Cryptosporidium* spp. infection in feline and canine fecal samples

Diagnostic methods	Result	Positive (*n*)	Negative (*n*)	Kappa	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	DFAT% (Positive/*n*)
ICT	Positive	6	3	0.53	46.1 (40.7–51.5)	99.0 (97.9–100)	0.66	0.97	2.7 (9/328)
	Negative	7	312						
PCR	Positive	5	2	0.48	38.4 (33.1–43.7)	99.3 (98.4–100)	0.71	0.97	2.1 (7/328)
	Negative	8	313						

n = 328

NPV: Negative predictive value; PPV: Positive predictive value; ICT: immunochromatography rapid test. DFA was used as gold standard. 95% Confidence Intervals (95% CI) are indicated

Table 5 Prevalence of *G. duodenalis* and *Cryptosporidium* spp. infection in dogs according to epidemiological and clinical variables

Variable	Samples (*n*)	*Giardia duodenalis*		*Cryptosporidium* spp.	
		Positive (%)	*p*-value	Positive (%)	*p*-value
Sex					
Male	130	43 (33.1)	0.27	7 (5.4)	0.42
Female	95	25 (26.3)		3 (3.2)	
Age group					
Puppy	55	22 (40.0)	0.14	6 (10.9)	0.058
Young	30	8 (26.7)		1 (3.3)	
Adult	123	36 (29.2)		3 (2.4)	
Senior	17	2 (11.8)		0 (0.0)	
Origin					
Collectivity	13	8 (61.5)	**0.04**	0 (0.0)	0.37
Owner	152	43 (28.3)		9 (6.0)	
Shelter	60	17 (28.3)		1 (1.7)	
Fecal consistency[a]					
1–2	6	1 (16.6)	0.22	0 (0.0)	0.8
3–4	122	32 (26.2)		5 (4.1)	
5–7	97	35 (36.1)		5 (5.2)	
Total	225	68 (30.2)		10 (4.4)	

[a]Fecal consistency according to the Bristol stool chart (see text)

DFA was used as gold standard

Statistically significant values are indicated in bold

Table 6 Prevalence of *G. duodenalis* and *Cryptosporidium* spp. infection in cats according to epidemiological and clinical variables

Variable	Samples (n)	*Giardia duodenalis*		*Cryptosporidium* spp.	
		Positive (%)	*p*-value	Positive (%)	*p*-value
Sex					
Male	60	6 (10.0)	0.53	0 (0.0)	**0.03**
Female	43	6 (14.0)		3 (7.0)	
Age group					
Kitten	17	0 (0.0)	0.17	1 (7.7)	0.18
Young	24	5 (20.8)		2 (8.3)	
Adult	58	6 (19.0)		0 (0.0)	
Senior	4	1 (25.0)		0 (0.0)	
Origin					
Collectivity	4	0 (0.0)	0.43	0 (0.0)	0.86
Owner	55	5 (9.1)		2 (3.6)	
Shelter	44	7 (16.0)		1 (2.3)	
Fecal consistency[a]					
1–2	15	2 (13.3)	0.82	1 (6.7)	0.45
3–4	68	7 (10.3)		1 (1.5)	
5–7	20	3 (15.0)		1 (5.0)	
Total	103	12 (11.7)		3 (2.9)	

[a]Fecal consistency according to the Bristol stool chart (see text)

DFA was used as gold standard

Statistically significant values are indicated in bold

Cryptosporidium spp. infection in human stool samples based on its optimal diagnostic performance, ease of use, and cost-effectiveness [43]. In addition, DFA allows enumeration and estimation of (oo)cyst concentrations in fecal specimens. Comparatively, much less information is currently available on the suitability of DFA as diagnostic tool in small animal veterinary practice. This is primarily because this method requires relatively expensive

equipment that is not always available in small veterinary clinics [47].

In this study, MIF delivered the lowest diagnostic sensitivity of the three compared methods (using DFA as standard) for the detection of *G. duodenalis*. This finding was somehow expected as low parasite burdens and/or intermittent cyst shedding are both known factors that impair the accuracy of microscopy detection [48]. In our study, an experienced parasitologist examined the samples, explaining why diagnostic specificity and positive and negative predictive values obtained by MIF were comparable to those obtained with the more expensive ICT and real-time PCR techniques. An added benefit of MIF is that this technique is recommended to be used in clinically ill animals because enables the identification of other parasitic forms or genera that might be present in the examined sample, including coccidia (*Cystoisospora* spp.) and helminthic (cestode, trematode, and/or nematode) eggs [3, 24]. While MIF is unsuited for the precise assessment of cyst viability based on morphological traits only, it has valued for the follow up of the infection or for post-treatment monitoring [49]. We did not assess the performance optic microscopy technique for the detection of *Cryptosporidium* spp. Due to the difficulty of detecting oocysts in the samples of feces of carnivores because of their small size (approx. 4 µm) and the fact that they can be shed intermittently in feces [50], therefore, a negative result using this method is not conclusive.

ICT kits have become popular for practitioners in veterinary clinics to make a first approach to the diagnosis of protozoan infections due to the simplicity of their procedure, the minimal resource requirements, and the rapidity of in-house results [16]. In our hands, ICT yielded a moderate (<70%) diagnostic sensitivity but the highest (97%) specificity among all compared techniques for the detection of *G. duodenalis* infection. Commercial ICT kits have variable sensitivity rates (range: 44–87%) depending on the animal population under study and its clinical status [30, 51, 52]. Of note, false-negative results are highly expected in samples with low cyst counts [46], a limitation previously reported for the commercial ICT kit used here [53]. Therefore, in negative samples of dogs with compatible clinical signs of giardiosis, it is recommended either to repeat the exam or proceed with further DFA or PCR testing. Because ICT is not a quantitative assay and obtained results require proper interpretation, this method should not be used in post-treatment giardiosis control analyses. Fecal antigen tests should be used as an addition to fecal flotation specially when evaluating dogs and cats with diarrhea. These tests are not recommended for use in clinically healthy pets or for monitoring therapy due to the uncertain clinical and zoonotic implications of a positive antigen result combined with a negative cyst presence result in a healthy pet. Additionally, the duration for which *Giardia* antigen assay results remain positive after the resolution of diarrhea is still unclear. Therefore, if a veterinarian chooses to assess the effectiveness of giardiosis treatment in cats and dogs, follow-up evaluations should be conducted using only optical microscopy methods [3].

Finally, real-time qPCR was the assay exhibiting the highest diagnostic sensitivity (78.7%) for the detection of *G. duodenalis* infection in this study. This value is considerably lower than that (98.1%) obtained in a similar survey also using DFA as gold standard [44]. In this survey, real-time PCR performed better than DFA for the detection of *G. duodenalis* infection in dogs (71 vs. 68, respectively) but not in cats (8 vs. 12, respectively). A positive PCR result does not always indicate active shedding or disease status, as pathogen nucleic acids can be present in subclinical infections or in animals that have recovered but still carry nonviable pathogens, free nucleic acids, damage cells, or debris. Additionally, positive PCR results may arise from environmental DNA contamination of specimens. Therefore, positive test results must be evaluated considering the patient's history, clinical signs, physical examination findings, the purpose of the test other diagnostic test results and an understanding of how often these organisms are found in healthy versus diseased animals [3].

These data indicate that both PCR and DFA provide similar diagnostic performance for the detection of the parasite. An additional advantage of PCR methods is that, when coupled with Sanger sequencing, they allow for the identification of the parasite´s genotype/sub-genotype. Although not useful in terms of the management of the infected animal, molecular data is essential in epidemiological investigations to assess sources of infection, transmission pathways and zoonotic potential. PCR platforms can also improve turnaround times in laboratory settings with high sample processing work loads [54].

As for the detection of *Cryptosporidium* infection, a low diagnostic sensitivity rate of 46.1% was obtained for ICT. This rate was similar to those (25-42.9%) reported in other canine and feline populations in previous studies using different commercial ICT kits [34, 55]. Taking together, these data suggest that ICT may not be the most reliable option for detecting *Cryptosporidium* infection in canine and feline fecal samples. Several reasons can account for the discrepancies observed in the diagnostic sensitivities yielded by different commercial ICT kits including (i) the clinical status (with or without clinical signs) of the canine/feline population investigated, (ii) the parasite burden, and therefore, the oocyst concentration in feces, and (iii) the fact that monoclonal antibodies directed against different sets of surface epitopes can affect the performance of the test. In this regard, it should be noted that most commercially available ICT kits are

specifically designed to detect zoonotic *Cryptosporidium parvum*, but not canine-adapted *Cryptosporidium canis* or feline adapted *Cryptosporidium felis* infections [55]. Therefore, appropriate validation procedures must be carried out to guarantee the correct performance of ICT kits in small animal veterinary clinics.

A surprisingly low diagnostic sensitivity rate of 38.4% was observed for the detection of *Cryptosporidium* spp. infection by PCR in this study. The reasons behind this low performance are unclear, but they might be related to (i) inadequate conservation of fecal samples or incomplete breakage of the oocysts´ wall during the DNA extraction process, leading to suboptimal amount of quality DNA [56], (ii) insufficient removal of PCR inhibitors (polysaccharides, bile, salts, lipids, urate) during the DNA purification process [57, 58], and (iii) low oocyst shedding in the feces [59].

Our comparative analysis of the diagnostic performance of MIF, ICT and PCR using DFA as gold standard revealed that MIF detected the lowest number of *Giardia*-positive samples. A practical approach for the detection of this parasite would be based on a preliminary screening based on ICT and subsequent confirmation of positive cases (or negative cases with compatible clinical signs) by DFA. In the case of *Cryptosporidium* detection, DFA should be regarded as the first-line detection method considering the low diagnostic sensitivities of ICT and Ziehl-Neelsen stain (the latter not tested in the present study).

In this study, the number of analyzed dog samples surpassed that of cat samples (68.6% vs. 31.4%, respectively). This proportion reflects what it is typically seen in routine veterinary practice, as dog owners visit clinics more frequently than cat owners and, therefore, feline samples are less readily available [60]. Indeed, it has been estimated that cat owners visit veterinary clinics with their pets up to 40% less frequently compared to dogs' owners [61].

The findings of this study revealed higher rates of *G. duodenalis* and *Cryptosporidium* spp. infection in dogs compared to cats, in line with previous studies [1, 21], observing a statistically significant association between *G. duodenalis* detection and dog species, corroborating findings from similar studies [62]. In contrast, no such association was detected for *Cryptosporidium* infection diagnosis, possibly due to the low prevalence of this parasite in dogs [4] and cats [19].

Our risk association analyses revealed that dogs from collectivities (this is, breeding and controlled feline colonies) were more likely to harbor *G. duodenalis* infections than owned and sheltered dogs. Overall, animals sharing household/environments tend to have higher infection rates because regular contact increases the likelihood of pathogen transmission [21, 63]. Low sample size for this specific canine subpopulation may account, at least partially, for this. On regards age variable, we did not find a statistically significant differences in between *Cryptosporidium* and *G. duodenalis* infection in dogs or cats. However, it is important to highlight the role of puppies/kittens in the transmission of parasitic infections, as they are the subpopulation at higher risk of infection and from a closer bond with their owners, potentially posing a risk to certain human group of risk such as young children, the elderly and immunocompromised individuals [11, 64].

Female cats were more likely to harbor *Cryptosporidium* infections than their male counterparts. Although numbers of positive cases were low and results should be interpreted with caution, this finding might be associated with reproductive behavioral or another epidemiological factors [65].

Limitations of the results of this study include the use only of fecal samples. Although it is a non-invasive sample, it also presents issues like the presence of PCR inhibitors such us complex polysaccharides, bile, salts, lipids and urate [57, 58]. Another influential factor is the time elapsed between sample collection and analysis, especially for samples from shelters, where it is difficult to ensure controlled shipping conditions. This can affect the (oo)cysts of *Cryptosporidium* and *G. duodenalis* [66]. Finally, the comparison could have included the use of more novel techniques like next-generation sequencing, which would have provided much more information but not be implemented due to cost issues [7].

Conclusions

DFA is the recommended method for routine diagnosis of *G. duodenalis* and *Cryptosporidium* spp. infection in canine and feline fecal samples in small animal practice based on its diagnostic performance and cost-effectiveness. However, the optical microscopy technique MIF is useful for decision making in post-treatment *G. duodenalis* infections.

Early and accurate diagnosis of both pathogens is important not only for timely treatment but also to prevent cross-species transmission of these parasites to humans and other animal hosts. The implementation of DFA (alone or combined with PCR when possible) in clinical and veterinary settings can significantly improve the diagnosis and control of these infections, thereby generating a positive impact on public health.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
J.P.B. participated in sampling and processing the fecal samples, carried out the microscopy, immunological and molecular procedures, performed the statistical analysis of the data and drafted and finalized the manuscript. A.M. & G.M. conceived and coordinated the study, participated in the diagnostic assays, assisted with data analysis and reviewed the final manuscript. D.C.: coordinated the molecular diagnosis assays and substantively revised the manuscript. C.F., J.S., V.M., E.E.-S. and R.C. processed the fecal samples and assisted with the performance of non-molecular diagnosis assays. BB participated in the molecular diagnosis assays. All authors read and approved the final manuscript.

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Data availability
Data is provided within the manuscript and in supplementary information files.

Declarations

Ethic approval and consent to participate
This study was carried out in accordance with Spanish legislation guidelines (DR 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).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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