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RESEARCH

Prevalence and temporal dynamics of *Cryptosporidium* spp., *Giardia duodenalis*, and *Blastocystis* sp. among toddlers attending day-care centres in Spain. A prospective molecular-based longitudinal study

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Abstract

Giardia duodenalis, *Cryptosporidium* spp., and *Blastocystis* sp. are common intestinal eukaryotic parasites affecting children in developed and resource-limited countries. Lack of information on the epidemiology and long-term stability in asymptomatic children complicates interpretation of transmission and pathogenesis. To assess the occurrence, genetic diversity, and temporal dynamics of intestinal eukaryotic parasites in young children, 679 stool samples from 125 toddlers attending six public day-care centres in Central Spain were collected bimonthly within a 1-year period. Detection and identification of species/genotypes were based on PCR and Sanger sequencing methods. Four eukaryotic species were identified: *G. duodenalis* (2.5–31.6%), *Cryptosporidium* spp. (0.0–2.4%), *Blastocystis* sp. (2.5–6.4%), and *Entamoeba dispar* (0.0–0.9%). *Entamoeba histolytica* and *Enterocytozoon bieneusi* were undetected. Sequence analyses identified assemblage A (63.6%) and B (36.4%) within *G. duodenalis* ($n=11$), *C. hominis* (40%), *C. parvum* (40%), and *C. wrairi* (20%) within *Cryptosporidium* spp. ($n=5$), and ST1 (3.8%), ST2 (46.2%), ST3 (15.4%), and ST4 (34.6%) within *Blastocystis* sp. ($n=26$). *Giardia duodenalis* sub-assemblage AII/AIII was detected in a toddler for 10 consecutive months. Stable carriage of *Blastocystis* ST2 allele 9, ST3 allele 34, and ST4 allele 42 was demonstrated in five toddlers for up to 1 year.

Conclusions: *Giardia duodenalis* and *Blastocystis* sp. were common in toddlers attending day-care centres in Central Spain. Long-term infection/colonization periods by the same genetic variant were observed for *G. duodenalis* (up to 10 months) and *Blastocystis* sp. (up to 12 months).

What is Known:
- *Asymptomatic carriage of G. duodenalis and Blastocystis sp. is frequent in toddlers.*
- *The epidemiology and long-term stability of these eukaryotes in asymptomatic young children is poorly understood.*

What is New:
- *Long-term colonization/infection periods by the same genetic variant were described for Blastocystis sp. (up to 12 months) and G. duodenalis (up to 10 months).*

Keywords *Cryptosporidium* · *Giardia* · *Blastocystis* · Toddlers · Chronicity · Genetic diversity · Genotyping

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Abbreviations

bg	β-Giardin
Ct	Cycle threshold
gdh	Glutamate dehydrogenase
gp60	60 KDa glycoprotein
ITS	Internal transcriber spacer
MED	Median
MLST	Multilocus sequence typing
qPCR	Real-time PCR

SD	Standard deviation
SNP	Single nucleotide polymorphism
ssu rRNA	Small subunit ribosomal RNA

Introduction

The intestinal eukaryotic parasites *Blastocystis* sp., *Giardia duodenalis*, and *Cryptosporidium* spp. are consistently detected (typical range: 1–18%) in paediatric patients [1–4]. In this context, infections with *G. duodenalis* and *Cryptosporidium* spp. in children are usually associated with acute self-limiting diarrhoea or chronic diarrhoea with or without malabsorption syndrome. Other common clinical manifestations are nausea, vomiting, abdominal pain, flatulence, and failure to gain weight [5, 6]. Long-term sequelae (particularly associated to *Giardia* infections) may include blood loss, anaemia, stunting, or impaired neurocognitive development [7–9]. Of note, subclinical infections by *Blastocystis* sp., *G. duodenalis*, and *Cryptosporidium* spp. are also common during childhood [10]. Sporadic outbreaks of giardiosis/cryptosporidiosis have been reported among children attending day-care centres [1, 11], after ingestion of contaminated water or food [12], in close contact with infected animals [13, 14], or using recreational waters [15, 16]. Although the pathogenic significance of *Blastocystis* sp. is still controversial, it has been primarily linked with chronic abdominal pain in children and teenagers [17]. Two outbreaks of gastrointestinal illness associated to *Blastocystis* infection have been reported to date [18, 19].

Progression from infection to disease is a multifactorial process involving variables associated to the pathogen (species/genotype, virulence, burden), the host (age, immune and nutritional status) and the interface between them (microbiota composition). Microeukaryotic enteroparasites may influence microbiota homeostasis and overall health. Whereas *Blastocystis* sp. has been primarily identified as a common component of the healthy gut microbiome [20], disrupted microbiota (disruption of the microbial biofilm structure, altered virulence in commensal species, altered species abundance/diversity) by *G. duodenalis* infection plays a role in *Giardia* pathogenesis [21]. In this context, depletion of the intestinal microbiota by antibiotic treatment may led to a consequent impact on the *Giardia* infection dynamics, e.g. by lowering intestinal motility and decreasing clearance of the parasite from the intestinal tract [22]. *Cryptosporidium* infections have also been linked with remodelling of the gut microbiota in murine and non-human primate hosts [23, 24].

Chronic infections by *Blastocystis* sp., *G. duodenalis*, and *Cryptosporidium* spp. are well documented in children and adult patients [25–27]. Molecular variability has been identified as a factor potentially involved in the establishment of chronic *Giardia* and *Blastocystis* infections [28, 29], whereas differentiation between long-term infection and re-infection is important for tailored treatment and management of patients infected with intestinal eukaryotic parasites [30].

There is little information on the genetic diversity and temporal dynamics of intestinal eukaryotic species in asymptomatic young children. Available prospective longitudinal studies assessing stability and acquisition/loss rates have only been conducted for *Blastocystis* sp. in healthy adults from Ireland [31] and healthy Dutch returning travellers [32], for *G. duodenalis* in infants and toddlers from Bangladesh [33] and Malawi [9], and for *Cryptosporidium* spp. in Malawian children hospitalized with diarrhoea [34]. To contribute bridging this gap of knowledge, this study aimed to investigate the genetic diversity, long-term presence, and stability of potential diarrhoea-causing intestinal protist eukaryotic parasites in toddlers attending day-care centres in Central Spain.

Materials and methods

Design

This is a prospective longitudinal, molecular-based study. A total of 125 toddlers (4—42 months of age; male/female ratio: 0.87) attending six public day-care centres in Majadahonda and Las Rozas (Central Spain) were investigated through a 12-month period (September 2020 to August 2021). Sampling was conducted sequentially every 2 months. All toddlers attending the surveyed day-care centres were invited to participate without any exclusion criteria. The median participation rate was 17.9% (range: 8.1–40.3%) of toddlers.

Sampling

Informative meetings were held for interested families, which were provided with sampling kits (sterile polystyrene plastic flask with spatula and a unique identification number) to obtain individual stool samples. Signed informed consents were obtained from parents/legal guardians, who assisted in collecting the stool samples from toddlers and brought them to day-care centres. Samples were transported to the Spanish National Centre for Microbiology by members of the research team at scheduled times (usually 2–3 days after kits were provided) and stored at 4 °C (1–5 days) or −20 °C (> 5 days) without preservatives until further diagnostic and molecular analyses. A total of 124 individual stool samples were collected in the first sampling period, 121 in the second, 112 in the third, 112 in the fourth, 110 in the fifth, and 98 in the sixth.

Questionnaire survey

A standardized questionnaire (Online Resource Table 1) was provided as part of the sampling kit to be completed by the toddler's parents/legal guardians. Questions included demographic characteristics (age, sex, country of birth, number of siblings), behavioural habits such as hand and fruit/vegetable washing, and occurrence of diarrhoea episodes and other clinical manifestations (abdominal pain, vomiting, nausea, reduced appetite) in the participant, family members, school mates, and/or pets. Additional questions addressed potential risk factors such as types of drinking water, swimming in pools or natural waters, contact with pets, and recent travel abroad. Completed questionnaires and signed informed consents were returned together with the stool samples as explained above.

DNA extraction

Genomic DNA was extracted from 200 mg of faecal material using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted and purified DNA samples in molecular grade water (200 µL) were kept at −20 °C until further analysis.

Molecular detection and characterization of *Giardia duodenalis*

Detection of *G. duodenalis* DNA was achieved using a qPCR method targeting a 62-bp fragment of the *ssu* rRNA gene [35]. Amplification reactions (25 µL) consisted of 3 µL of template DNA, 0.5 µM of the primer set Gd-80F/Gd-127R, 10 pmol of probe, and 1X TaqMan® Gene Expression Master Mix (Applied Biosystems, CA, USA).

A multilocus sequence typing scheme based on the amplification of partial sequences of the *gdh*, *bg*, and *tpi* genes of *G. duodenalis* was used for genotyping purposes. A semi-nested PCR targeting a 432-bp fragment of the *gdh* gene was performed in 25 µL reaction mixtures including 5 µL of template DNA and outer (GDHeF/GDHiR) and inner (GDHiF/GDHiR) primer sets [36]. A nested PCR was used to amplify a 511-bp fragment of the *bg* gene in 25 µL reaction mixtures including 3 µL of template DNA and outer (G7/G759) and inner (G99/G609) primer pairs [37]. A nested PCR was also used to amplify a 530-bp fragment of the *tpi* gene. Reaction mixtures (50 µL) included 2−2.5 µL of template DNA and outer (AL3543/AL3546) and inner (AL3544/AL3545) primer pairs [38].

Molecular detection and characterization of *Cryptosporidium* species

Cryptosporidium spp. was detected using a nested PCR amplifying a 587-bp fragment of the *ssu* rRNA gene [39]. Reaction mixtures (50 µL) for both reactions included 3 µL of template DNA and outer (CR-P1/CR-P2) and inner (CR-P3/CPB-DIAGR) primer sets. Sub-typing of the isolates identified as *C. hominis*/*C. parvum* was attempted at the *gp60* gene using the AL-3531/AL-3535 and AL-3532/AL-3534 primer pairs [40].

Molecular detection of *Blastocystis* species

Detection of *Blastocystis* sp. was accomplished by a direct PCR targeting a 600-bp fragment of the *ssu* rRNA gene [41]. Reaction mixtures (25 µL) included 5 µL of template DNA and the pan-*Blastocystis*, barcode primers RD5 and BhRDr.

Molecular detection of *Enterocytozoon bieneusi*

Enterocytozoon bieneusi was detected using a nested PCR amplifying a 390-bp fragment including the entire ITS and portions of the flanking large and small subunit of the rRNA gene [42]. Reaction mixtures (50 µL) included 1 µL of template DNA and outer (EBITS3/EBITS4) and inner (EBITS1/EBITS2.4) primer sets.

Molecular detection of *Entamoeba histolytica* and *Entamoeba dispar*

Differential diagnosis between pathogenic *E. histolytica* and non-pathogenic *E. dispar* was achieved by qPCR targeting a 172-bp fragment of the *ssu* rRNA gene [43, 44]. Reaction mixtures (25 µL) included 3 µL of template DNA and the *E. histolytica/E. dispar*-specific primers Ehd-239F and Ehd-88R.

General procedures for the molecular detection and sequencing of enteric protist parasites

All the direct, semi-nested, and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems). qPCR protocols were performed on a Corbett Rotor Gene™ 6000 real-time PCR system (QIAGEN, Hilden, Germany). 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 visualized 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. Primer and probe sequences used in the qPCR and conventional PCR protocols described above are shown in Online Resource Table 2. Positive-PCR products were directly sequenced in both directions using internal primer sets by capillary electrophoresis using the BigDye Terminator chemistry (Applied Biosystems) on an on ABI 3730xl automated DNA sequencer.

Molecular detection of other enteric bacterial and viral pathogens

The presence of enteric bacterial (enteroaggregative *Escherichia coli*, verocytoxigenic *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*/*Shigella*, *Aeromonas*, *Campylobacter*, *Clostridioides difficile*, *Salmonella*, *Vibrio*, *Yersinia*) and viral (astrovirus, norovirus, rotavirus) pathogens was also investigated by PCR methods. Detailed information on these agents will be provided in an independent study.

Sequence analyses

Raw sequencing data were viewed using the Chromas Lite version 2.1 sequence analysis program (Technelysium Pty Ltd., South Brisbane, Australia). Generated DNA consensus sequences were aligned to appropriate reference sequences retrieved from the NCBI GenBank database using the MEGA version 6 software [45]. *Blastocystis* sequences were submitted at the *Blastocystis* 18S database (http://pubmlst.org/blastocystis/) for sub-type confirmation and allele identification. Sequences generated in the present study have been deposited in GenBank under accession numbers OL632299–OL632301 and OL632303–OL632311 (*G. duodenalis*), OL638491–OL638494 (*Cryptosporidium* spp.), and OL623670–OL623673 (*Blastocystis* sp.).

Statistical analyses

Factors associated with a positive *G. duodenalis* result were examined using two models. For the fixed effects model, a main dataset was constructed with data from one of the six sampling periods—if the observation ever tested positive for *G. duodenalis*, we used data from the sampling point of the first positive *G. duodenalis* result; otherwise, we used data from the first sampling point in order. Potential risk associations with *P* values less than 0.05 from the univariable analysis were selected in the multivariable logistic regression model, using Akaike's information criterion and likelihood ratio tests to determine selection and evaluate the final model. Additionally, to account for periods where individuals were exposed to other *Giardia*-positive individuals in their day-care centre (i.e. high-risk periods), we conducted a random effects model by using a sample level dataset, excluding individuals with more than one *Giardia*-positive result during the study period. Analyses were performed using Stata version 17 (STATA Corp., College Station, TX, USA).

Results

A total of 677 stool samples from 125 toddlers were collected at bimonthly intervals within a 1-year period and subsequently analysed for the presence and molecular diversity of intestinal eukaryotic species. The full dataset showing the molecular (PCR and sequencing) data generated in the present study by individual toddler, participating day-care centre, and sampling period can be found in Online Resource Table 3. Overall participating rates decreased steadily along the course of the study from 96.8% in sampling period 2 to 78.4% in sampling period 6.

Overall, 52.0% (65/125) of toddlers were infected by *G. duodenalis* in at least one sample period, followed by *Blastocystis* sp. (5.6%, 7/125), *Cryptosporidium* spp. (4.0%, 5/125), and *E. dispar* (0.8%, 1/125). According to sampling period, frequencies of detection varied from 2.5−31.6% (MED: 14.0%; SD: 9.0%) for *G. duodenalis*, 0.0−2.4% (MED: 0.4%; SD: 0.9%) for *Cryptosporidium* spp., 2.5−6.4% (MED: 3.6%; SD: 1.2%) for *Blastocystis* sp., and 0.0−0.9% (MED: 0.4%; SD: 0.4%) for *E. dispar*. *Entamoeba histolytica* and *E. bieneusi* were undetected. Both *G. duodenalis* and *Blastocystis* sp. were identified in all six sampling periods, whereas *Cryptosporidium* spp. and *E. dispar* were detected only in three of the six sampling periods (Table 1).

Giardia duodenalis was identified in 65 toddlers. Most of them (66.2%, 43/65) were detected at single sampling periods; two toddlers (3.1%, 2/65) remained infected in five of the six sampling periods (Table 2). The parasite was reported in all six participating day-care centres at three or more sampling periods. Frequencies of detection varied greatly (range: 0.0−50.0%) both at the participating institution and sampling period levels (Fig. 1, panel a). Five toddlers (one girl, four boys) ≤ 2 years old were sporadically infected (≤ 1%) by *Cryptosporidium* spp. at single sampling periods. None of them presented with diarrhoea. The parasite was identified in four day-care centres during sampling periods 1, 2, and 4. *Entamoeba dispar* was detected in a single toddler during the first 6 months of study (sampling periods 1, 2, and 3). *Blastocystis* sp. was observed in seven toddlers (three girls, four boys) of all ages attending four day-care centres in at least five of the six sampling periods. Three

Table 1 Frequency of detection of intestinal eukaryotic species according to sampling period in toddlers attending day-care centres, Majadahonda (Spain)

	Sampling period											
	1 ($n=124$)		2 ($n=121$)		3 ($n=112$)		4 ($n=112$)		5 ($n=110$)		6 ($n=98$)	
Species	Pos	%	Pos	%	Pos	%	Pos	%	Pos	%	Pos	%
G. duodenalis	11	8.9	3	2.5	20	17.9	16	14.3	15	13.6	31	31.6
Cryptosporidium spp.	3	2.4	1	0.8	0	0.0	1	0.9	0	0.0	0	0.0
Blastocystis sp.	4	3.2	3	2.5	4	3.6	4	3.6	7	6.4	4	4.1
E. bieneusi	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
E. histolytica	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
E. dispar	1	0.8	1	0.8	1	0.9	0	0.0	0	0.0	0	0.0

toddlers (42.9%, 3/7) carried *Blastocystis* during the whole study period. Frequencies of detection (range: 0.0−25.0%) remained relatively stable through the sequential sampling in the four institutions where *Blastocystis* was circulating (Fig. 1, panel b). Most (92.6%, 25/26) of the stool samples with a *Blastocystis*-positive result were non-diarrhoeal.

Successful genotyping of *G. duodenalis* isolates was achieved in 12.4% (12/97) of samples with a positive result by qPCR. Most (82%, 9/11) of the isolates genotyped had qPCR Ct values ≤ 30. Molecular analyses revealed the presence of sub-assemblage AII (45.4%, 5/11), ambiguous AII/AIII sequences (18.2%, 2/11), sub-assemblage BIV (27.3%, 3/11), and an additional assemblage B sequence (9.1%, 1/11) of unknown sub-assemblage (Table 3). Three *Cryptosporidium* species were identified, namely *C. hominis* (40%, 2/5), *C. parvum* (40%, 2/5), and *C. wrairi* (20%, 1/5) (Table 3). All five *Cryptosporidium* spp. isolates failed to be amplified at the *gp60* loci, so their genotype family remains unknown. A thorough description of the *C. wrairi* case has been published elsewhere [46]. The *Blastocystis* subtypes identified were assigned to ST1 (3.8%, 1/26), ST2 (46.2%, 12/26), ST3 (15.4%, 4/26), and ST4 (34.6%, 9/26) (Table 3). The detailed molecular features (subtyping data, reference sequences, SNPs, GenBank accession numbers) of the representative *G. duodenalis*, *Cryptosporidium* spp., and *Blastocystis* sp. isolates characterised here are provided in Online Resource Table 4.

Based on the molecular data described above, the temporal stability of *G. duodenalis* and *Blastocystis* sp. in our paediatric cohort was further investigated (Fig. 2). *Giardia duodenalis* sub-assemblage AII/AIII was detected in a toddler attending day-care centre 2 for 10 consecutive months. The infection was lost or misdiagnosed at sampling period 6. *Blastocystis* ST2 allele 9 was identified in two toddlers attending day-care centres 2 and 4, respectively, along the whole study period. Similarly, *Blastocystis* ST4 allele 42 carriage was a stable feature in a toddler attending day-care centre 6. This very same genetic variant was acquired by another toddler of the same institution at sampling period 5 and maintained at sampling period 6. *Blastocystis* ST3 allele 34 was identified in a toddler attending day-care centre 3 for 10 consecutive months, being undetected at sampling periods 2 and 6, when infection was lost or misdiagnosed. Finally, all three *E. dispar*-positive samples corresponded to a toddler attending day-care centre 3 that carried the protozoan for six consecutive months and lost the infection at sampling period 4.

Table 2 Toddlers testing positive for *G. duodenalis*, *Cryptosporidium* spp., and *Blastocystis* sp. in one or more sampling period, Majadahonda (Spain)

Positive toddlers in one or more sampling periods	*Giardia duodenalis*		*Cryptosporidium* spp.		*Blastocystis* sp.	
	n	%	*n*	%	*n*	%
1 sampling period	43	66.2	5	100	2	28.6
2 sampling periods	17	26.2	0	0.0	1	14.3
3 sampling periods	3	4.6	0	0.0	0	0.0
4 sampling periods	0	0.0	0	0.0	1	14.3
5 sampling periods	2	3.1	0	0.0	0	0.0
6 sampling periods	0	0.0	0	0.0	3	42.9
Total toddlers	65	100	5	100	7	100

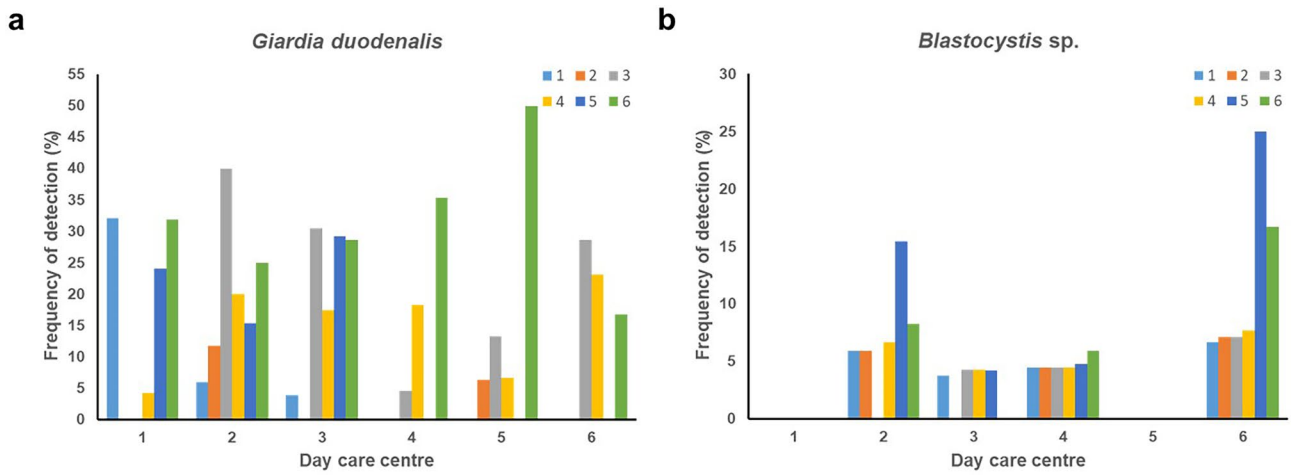

Fig. 1 Frequency of detection of intestinal eukaryotic species in children by day-care centre and sampling period, Majadahonda and Las Rozas (Spain). **a** *Giardia duodenalis*; **b** *Blastocystis* sp

Risk association analysis

The full dataset used for risk association analyses can be found in Online Resource Table 5. Out of the four micro-eukaryote species identified in the paediatric population under investigation, we identified variables associated with an increased risk of infection only for *G. duodenalis*.

Comparing G. duodenalis negative/ever positive

Overall, 53.8% of individuals who tested positive for *G. duodenalis* were male, majority were 2 years old, with none less than 1 year old (Online Resource Table 6). The most frequent symptom reported was diarrhoea (19.0%). For individuals positive for *G. duodenalis*, 3.1% were co-infected with enteropathogenic *E. coli* (EPEC) and *Clostridium difficile*, 10.8% EPEC only, 14.2% *C. difficile* only, and 4.6% with *Blastocystis* sp. at their first positive. One individual had recurrent enterotoxigenic *E. coli* (ETEC) and *G. duodenalis* (at 2 sampling periods).

In univariable analysis, older age, swimming, and number of samples were positively associated with *G. duodenalis* (Online Resource Table 6). The multivariable model retained age and swimming and showed that swimming was positively associated with higher odds of a G. *duodenalis* positive result [adjusted odds ratio (aOR) 5.67, 95% confidence interval (95% CI) 1.18–27.27]. However, in a random effects model, having another toddler with *G. duodenalis* in the day-care centre gave an aOR of 16.53 (95% CI 7.61–35.90). Swimming and age was no longer statistically significant, and there were no other infections associated with a *Giardia*-positive result (Online Resource Table 7).

Table 3 Genetic diversity of *Giardia duodenalis*, *Cryptosporidium* spp., and *Blastocystis* sp. identified in children attending public day-care centres, Majadahonda (Spain)

Species	Species/genotype	Sub-genotype	No. isolates	Relative frequency (%)
Giardia duodenalis	A	AII	5	45.4
		AII/AIII	2	18.2
	B	BIV	3	27.3
		Unknown	1	9.1
Cryptosporidium	*C. hominis*	Unknown	2	40
	C. parvum	Unknown	2	40
	C. wrairi[a]	Unknown	1	20
Blastocystis sp.	ST1	Allele 4	1	3.8
	ST2	Allele 9	12	46.2
	ST3	Allele 34	4	15.4
	ST4	Allele 42	9	34.6

[a]Please see reference [46]

Fig. 2 Temporal stability of *Giardia duodenalis* (light green figures) and *Blastocystis* sp. (light blue figures) according to sampling period in children attending day-care centres, Majadahonda and Las Rozas (Spain)

Individuals with serial results

When considering individuals with repeated samples (123 individuals had more than one sample), 47.2% (58/123) individuals were always negative, 35.0% (43/123) once *Giardia* positive, 0.8% (1/123) *Giardia* positive at all sampling periods they were tested, and 13.0% (16/123) discontinuously positive. 4.9% (6/123) remained positive after first positive. As such, among observations ever positive for *G. duodenalis*, 9.2% (6/65) remained positive after first positive. Of those continuously positive ($n=6$), five did not swim, only four reported drinking tap water and two also had EPEC. Three individuals who had more than one positive reported diarrhoea at first positive. One individual went on to report diarrhoea, abdominal pain, nausea, and vomiting and reduced appetite on subsequent positive, which was the next sampling period, which may be explained by their co-infection with norovirus.

Discussion

Microbial eukaryotes are common inhabitants of the human gut and include parasitic (pathogenic), not harmful (commensal), or beneficial (mutualist) taxa [47]. However, this distinction is not categorical. For instance, *Cryptosporidium* spp., *G. duodenalis*, and *Blastocystis* sp. can cause a wide range of clinical manifestations ranging from asymptomatic to chronic diarrhoea in school-age children in industrialized settings [48, 49]. Current evidence suggests that the outcome of infections by these microorganisms is the consequence of complex interactions between them, the gut bacterial and archaeal microbiota and the host immunity [50]. Indeed, some authors have suggested that *Blastocystis* sp. and *G. duodenalis* should be better regarded as commensals [51–53] or even pathobionts (this is, microorganisms that can cause or promote disease when specific genetic or environmental conditions are altered in the host) [54].

Molecular tools can assist on the differentiation between long-term infection (by the same strain) and re-infection (by a different strain) events. Few surveys (most of them were based on microscopy examination or enzyme immunoassays) have attempted to investigate this issue by analysing longitudinal data [55, 56]. Long-term temporal stability has been particularly studied for *Blastocystis* sp. For instance, four healthy adults from Ireland were consistently positive for the same *Blastocystis* subtype (determined at allele level) from 6 to 10 years [31]. In contrast, *Blastocystis* carriage has been deemed relatively short-lived in Dutch returning travellers [32] and treated children presenting with gastrointestinal manifestations in Turkey [55] and Switzerland [56]. In the present survey, *Blastocystis* carriage in toddlers (≤ 3 years) was consistently detected at low (2.5−6.4%) rates through the whole study period. These figures are considerably lower than those (10−13%) reported previously in older asymptomatic schoolchildren (4−14 years) from the same geographic area [49, 57]. This finding supports the hypothesis that the presence of *Blastocystis* is positively associated with age, with colonization being more common in older children and adults [57, 58]. Remarkably, three toddlers carried the same *Blastocystis* STs and allelic variants for a span of at least 12 months, whereas acquisition or loss of the protist was detected in two additional toddlers. Taken together, these data indicate that long-term host colonization is a stable

Blastocystis trait, also explaining the age-associated distribution of the protist mentioned above.

Available information on the natural history of *G. duodenalis* and *Cryptosporidium* spp. infections comes primarily from longitudinal birth cohort studies conducted in low-income settings. Hence, 55% of urban Bangladeshi infants had at least three *Giardia*-positive stools over the first 2 years of age [33]. Rates up to 77% have been reported for *Cryptosporidium* infections in slum-dwelling Bangladeshi [59] and Indian [60] children. A *Giardia* infection rate of 33%, rarely associated with illness, has been reported in toddlers attending a day-care centre in the USA [61]. In the present study, *Giardia duodenalis* was the most common enteric eukaryote identified (0−7%), whereas *Cryptosporidium* infections were only sporadically found (≤ 1%). These data agree with those (*Giardia*: 14−18%; *Cryptosporidium*: 1−4%) previously reported in pre- and schoolchildren from the same province [49, 57, 62]. Regarding temporal stability, most (67%) *Giardia* and all *Cryptosporidium* infections were identified at single sampling periods only, strongly suggesting that asymptomatic infections by these pathogens were resolved in relatively short periods. Despite this clear trend, it should be highlighted that two toddlers harboured subclinical *G. duodenalis* infections by up to 10 months. One of them harboured the same genetic variant during that period, indicating a probable long-term carriage and not re-infection. We have also shown in this study that G. *duodenalis* infection was related to swimming and age, but having other *Giardia* infected toddlers within a day-care centre was the main driver in increasing the risk of G. *duodenalis* infection.

Entamoeba histolytica and *E. bieneusi* were undetected in the surveyed toddler population. This is the first molecular-based study investigating the presence of *E. histolytica* in Spanish young children, whereas the absence of *E. bieneusi* confirms the results obtained in previous surveys targeting paediatric populations in the Madrid area [49, 57].

Genotyping analysis revealed the predominance of assemblage A (58%) over assemblage B (42%) in *G. duodenalis* isolates. This is in contrast with previous molecular data in asymptomatic children from the same area, where assemblage B (83−100%) was more prevalent than assemblage A (0−17%) [54, 57, 62]. Interestingly, three *Cryptosporidium* species (*C. hominis*, *C. parvum*, and *C. wrairi*) were found in the surveyed toddler cohort. This agrees with previous results in healthy children of paediatric age in the Madrid area, where *C. hominis* (71−100%) was more prevalent than *C. parvum* (0−21%) [54, 57]. The full account of the *C. wrairi* infection case has been provided elsewhere [46]. Results on the molecular diversity and frequency of *Blastocystis* subtypes were also expected, with ST2 (46%) being the most prevalent subtype identified, followed by ST4 (35%), ST3 (15%), and ST1 (4%). Very similar results were documented in other pre- and schoolchildren's populations in this very same geographical area [54, 57].

The results obtained in this longitudinal study may have been potentially biased by some methodological issues. Relatively low numbers of day-care centres investigated and toddlers recruited might not be representative of the whole sampling area. Because the study was based on voluntary participation, it is possible that families feeling at higher risk of infection were more prone to participate, distorting the final results. Another potential confounder could be reporting bias by parents during questionnaire completion. Finally, low amplification success rates of *Giardia*-positive samples in genotyping PCR assays and absence of data linking the presence of this parasite with the nutritional status of the toddlers investigated may have compromised the accuracy of some of our results.

In conclusion, we provided here longitudinal, molecular-based data indicating that (mostly asymptomatic) infections by *G. duodenalis* and *Blastocystis* sp., but not *Cryptosporidium* spp., are relatively common among toddlers attending day-care centres in central Spain. *Blastocystis* primarily presented as long-term colonization by the same genetic variant of the protist, supporting its commensal nature. In contrast, *G. duodenalis* and *Cryptosporidium* spp. tended to present as short-lived, self-limiting infections without the need of specific management, although subclinical *G. duodenalis* carriage can persist up to 10 months. Diagnosis of these microeukaryotic enteroparasites should be considered in cases with prolonged diarrhoea, although studies in apparently healthy paediatric populations might be useful to determine asymptomatic carriage rates and prevent infections to individuals at risk including immunocompromised patients and the elderly.

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Authors' contributions Sergio Sánchez and David Carmena contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Carolina Hernández-Castro, Alejandro Dashti, Sooria Balasegaram, Pamela Carolina Köster, Begoña Bailo, Andrea López, María Teresa Llorente, and David González-Barrio. The first draft of the manuscript was written by Carolina Hernández-Castro and David Carmena, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and material The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Code availability Not applicable.

Declarations

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Health Institute Carlos III (Date: April 4, 2019/CEI PI 11_2019-v2).

Consent to participate Written informed consent was obtained from the parents/legal guardians.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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