



Diagnosis of intestinal parasites in a rural community of Venezuela: Advantages and disadvantages of using microscopy or RT-PCR



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ABSTRACT

A cross-sectional study was carried out to determine the prevalence and diagnostic performance of microscopy and real time PCR (RT-PCR) for 14 intestinal parasites in a Venezuelan rural community with a long history of persistent intestinal parasitic infections despite the implementation of regular anthelmintic treatments. A total of 228 participants were included in this study. A multiplex RT-PCR was used for the detection of *Dientamoeba fragilis*, *Giardia intestinalis*, *Cryptosporidium* sp. and a multiplex RT-PCR for *Entamoeba histolytica*. Furthermore, a multiplex PCR was performed for detection of *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Necator americanus* and *Ancylostoma duodenale*. Combined microscopy-PCR revealed prevalences of 49.3% for *A. lumbricoides*, 10.1% for *N. americanus* (no *A. duodenale* was detected), 2.0% for *S. stercoralis*, 40.4% for *D. fragilis*, 35.1% for *G. intestinalis*, and 7.9% for *E. histolytica/dispar*. Significant increases in prevalence at PCR vs. microscopy were found for *A. lumbricoides*, *G. intestinalis* and *D. fragilis*. Other parasites detected by microscopy alone were *Trichuris trichiura* (25.7%), *Enterobius vermicularis* (3.4%), *Blastocystis* sp. (65.8%), and the non-pathogenic *Entamoeba coli* (28.9%), *Entamoeba hartmanni* (12.3%), *Endolimax nana* (19.7%) and *Iodamoeba bütschlii* (7.5%). Age- but no gender-related differences in prevalences were found for *A. lumbricoides*, *T. trichiura*, *G. intestinalis*, and *E. histolytica/dispar*. The persistently high prevalences of intestinal helminths are probably related to the high faecal pollution as also evidenced by the high prevalences of non-pathogenic intestinal protozoans. These results highlight the importance of using sensitive diagnostic techniques in combination with microscopy to better estimate the prevalence of intestinal parasites, especially in the case of *D. fragilis* trophozoites, which deteriorate very rapidly and would be missed by microscopy. In addition, the differentiation between the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* can be attained. However, microscopy remains an important diagnostic tool since it can detect other intestinal parasites for which no PCR is available.

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1. Introduction

Soil-transmitted helminth (STH) infections are one of the most important public health problems in tropical countries (de Silva et al., 2003; Hotez et al., 2006, 2008). It is estimated that they affect approximately 1.4 billion people worldwide (Dunn et al., 2016), with *Ascaris lumbricoides*, *Trichuris trichiura* and hook-

worms being accountable for respectively 819, 465 and 439 million infections in 2010 (Pullan et al., 2014). Regarding protozoan parasites, those most commonly associated with diarrhoea in humans are *Giardia intestinalis*, *Entamoeba histolytica*, *Coccidia* and *Dientamoeba fragilis* (Amin, 2002; Bethony et al., 2006; Verweij and van Lieshout, 2011). Several outbreaks of diarrhoeal disease have been reported in community settings in the developed world, especially for parasitic protozoa transmitted with water (MacKenzie et al., 1995; Stephenson et al., 2000; Ashbolt, 2004; Karanis et al., 2007; Baldursson and Karanis, 2011).

Field data on parasitic infections in developing countries are crucial to assess the magnitude of the problem. The diagnosis of

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intestinal parasitic infections is typically performed using microscopic examination of stool samples, which has the advantages of being largely affordable, relatively easy to perform in resource-limited settings, and able to detect several parasites of clinical significance. Additionally, microscopy can reveal the presence of pathogenic, as well as non-pathogenic, parasites that are not usually searched for using generally more sensitive methods like PCR. However, the use of microscopy has also some disadvantages, one of which is that trophozoites of protozoan parasites, particularly *D. fragilis*, tend to deteriorate rapidly outside the host, meaning that they are to be found only in those samples that are examined right after collection. Another problem is that intestinal protozoa can easily be missed due to day-to-day variation in shedding. Comparative studies indicate that for reliable diagnosis of intestinal protozoan infections, the use of a fixative and multiple sampling is required in order to enhance the diagnostic quality (Hashmeyer et al., 1997). Indeed to mitigate the risk of under-diagnosis, a modified Triple Faeces Test (TFT) has been recommended to significantly improve the detection sensitivity (van Gool et al., 2003; Vandenberg et al., 2006). This test combines the use of fixatives and multiple sample collection for three consecutive days. The use of real-time PCRs is an alternative (ten Hove et al., 2007), although its use in developing countries is still limited by the relatively high costs (Haque et al., 2007). Multiplex PCR simultaneously detects different parasites in a single reaction and is less dependent on the skills of the laboratory staff.

In this study, both microscopy and real-time PCR were used in a comparative fashion to determine the prevalence of several intestinal helminth and protozoan parasites in a rural community in the central-north region of Venezuela.

2. Materials and methods

2.1. Study population

This study was conducted in a rural community called “Caserio El 25”, which is located in the central-north region of Venezuela, in the municipality of “Carlos Arvelo” and “Parroquia Tacarigua”, at about 25 km south-east from the city of Valencia.

2.2. Ethical aspects

This study was part of a larger project intending to elucidate and evaluate the prevalence and risk factors of helminth infections in the rural community “Caserio El 25”, Carabobo State, Venezuela. The objectives of the project were explained to the members of each household in the community to obtain a written informed consent from adults and parents and custodian of children. The study adheres to local ethical criteria (Ethical Committee of the Carabobo State Health Authority, INSALUD), and was approved by the ethical committee of VU University, Amsterdam. At the end of the study, all participants were offered (free of charge) a single dose of Pirantel® as deworming agent.

2.3. Methodological characterization of the work

A cross-sectional study was carried in the studied community in April 2010. All the 470 inhabitants of the community were asked to participate, without pre-established exclusion criteria. However, in order for the participants to be included, it was required that three faecal samples were provided.

2.4. Sampling

Stool specimens were collected according to a modified routine procedure known as the triple faeces test (TFT). Individuals were

then asked to fill 3 tubes with faecal material on three consecutive days. One tube was preserved with ethanol for molecular analysis and the two other tubes were filled with sodium acetate, acetic acid and formalin (SAF) for microscopic examination (ten Hove et al., 2007). Faecal samples for molecular analysis were stored at -20°C until DNA extraction. SAF preserved samples were stored at 4°C . In total, 228 participants were then included in the study. Individuals excluded were either those lost to follow up or were unable to provide three adequate samples.

2.5. Microscopy

SAF-preserved stool samples were microscopically examined for the presence of intestinal protozoa and helminths (trophozoites, cysts, eggs and larvae). Samples were examined using both a direct/wet-mount and a smear Iron Haematoxylin Kinyoun (IHK) permanent stain preparation, which combines staining of haematoxylin dye and carbol-fuchsin (Ziehl Neelsen staining). Each of two consecutive faecal samples per person was examined with one wet and one stained slide.

2.6. DNA extraction from faecal specimens

In order to isolate DNA, 300 μl of ethanol-preserved faeces suspension was centrifuged for 1 min (10,000 rpm) and the pellet washed with 1 ml 1x PBS. After centrifugation, the pellet was resuspended into 200 μl of freshly prepared lysis buffer (2% polyvinylpyrrolidone, 10% SDS, 0.5 M EDTA) and heated for 10 min at 100°C . After proteinase K (Roche, Switzerland) treatment (2 h at 55°C), DNA was extracted using phenol-chloroform protocol. After centrifugation (8000 rpm), the upper phase was mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (pH 8.0). Samples were mixed in a Vortex for 30 s at full speed, and the aqueous phase containing nucleic acids was separated by centrifugation (12,000 rpm) for 5 min. The aqueous phase was transferred in new vials, and phenol was removed by mixing with an equal volume of chloroform-isoamyl alcohol (24:1) followed by repeated vortex and centrifugation (12,000 rpm) for 5 min. The supernatant was transferred to a fresh tube and one volume of isopropanol was added and left at -20°C overnight to precipitate the DNA. After centrifugation (10,000 rpm) for 10 min, the supernatant was discarded and the DNA pellet was dried. After washing with 70% ethanol, DNA was resuspended in 100 μl of Tris-EDTA (10 mM Tris.HCl pH7.4, 1 mM EDTA pH 8.0).

2.7. Primers and probes

All primers and probes used in this study were purchased from Biolegio (Holland). Table 1 shows the PCR primers and taqman probes. *D. fragilis* (GenBank accession number U37461), *G. intestinalis* (GenBank accession number M54878), and *E. histolytica* (GenBank accession number X64142, Z49256) primers and probes were designed by Verweij et al. (2004). The *Cryptosporidium* sp. (accession number AF188110) specific primers and taqman detection probe were described by Fontaine and Guillot (2002). PCR of the nematodes *A. lumbricoides*, hookworms (*A. duodenale* and *N. americanus*) and *S. stercoralis* was also performed in a multiplex PCR with primers and probes described by Basuni et al. (2011).

2.8. PCR amplification and detection assay

For detection of inhibition during the amplification, specific primers and probes for the phocine Herpes Virus (PhHV-1) were included in the assay as internal control to monitor potential faecal contaminants (Basuni et al., 2011). Amplification of the PhHV-1 internal control was, by definition, detected within the correct

Table 1
Primers and probes for real time PCR assays of helminths and protozoa.

Organism	Primer and probes	Sequence (5'-3') label and quencher
<i>A. lumbricoides</i>	Alum96F Alum183R Alum124T-TP	GTAATAGCAGTCGGCGGTTTCTT GCCCAACATGCCACCTATTC Vic-TTGGCGGACAATTGCATGCGAT-BHQ1
<i>A. duodenale</i>	Ad125F Ad195R Ad155MGB-TP	GAATGACAGCAAACCTGTTGTTG ATACTAGCCACTGCCGAAACGT Fam-ATCGTTTACCGACTTTAG-BHQ1MGB
<i>N. americanus</i>	Na58F Na158R Na-TP	CTGTTTGTGCAACGGTACTTGC ATAACAGCGTGCACATGTTGC Tex Red-GTGTTCAGCAATTCCTGTTAAGTGAAG-BHQ2
<i>S. stercoralis</i>	Stro18s-1530F Stro18s-1630R Stro TP	GAATCCAAGTAAACGTAAGTCATTAGC TGCCTCTGGATATTGCTCAGTTC ATTO425-ACACACCGCGCTCGCTGC-BHQ1
<i>G. intestinalis</i>	Giardia-80F Giardia-127R Giardia-tp	GACGGCTCAGGACAACGGTT TTGCCAGCGGTGTCCG FAM-CCCGCGCGGTCTCTGCTAG-BHQ1
<i>D. fragilis</i>	Df-124F Df-221R Df-172	CAACGGATGCTTGGCTCTTTA TGCATTCAAAGATCGAATTCATCAC VIC-CAATTCTAGCCGCTTAT-BHQ1
<i>E. histolytica</i>	Ehd-239F Ehd-88R Histo-tp	ATTGTCGTGGCATCCTAACTCA GCGGACGGCTCATTATAACA Texas Red-TCATTGAATGAATTGGCCATT
<i>Cryptosporidium</i> sp.	Cr-F Cr-R Cr-tp	CGCTTCTCTAGCCTTTTCATGA CTTCAGTGTGTTTGCCAAT Texas Red-CCAATCACAGAATCATCAGAATCGACTGGTATC-BHQ2
PhHV-1	PhHV-F PhHV-R PhHV-tp	GGGCGAATCACAGATTGAATC GCGGTTCCAAACGTACCAA CY5-TTTTATGTGTCCGCCACCATCTGGATC-BHQ2

crossing point values (CP) range for all samples. No samples were excluded due to inhibition, and all ethanol fixed samples from EI 25 were assayed using PCR.

Amplification of *E. histolytica* was performed in a volume of 20 μ l with Roche PCR master mix, 0.70 pmol of each *E. histolytica* primer, 0.5 pmol of each PhHV-1 specific primer, 0.15 pmol of *E. histolytica* probe, 0.75 pmol of PhHV-1 probe, and 5 μ l of 1:20 DNA diluted sample. Dilutions were made with TE buffer and PhHV-1 was added to serve as an internal control for amplification. For amplification of *D. fragilis*, *Cryptosporidium* sp. and *Giardia intestinalis* DNA, a multiplex real time PCR was performed in a volume of 25 μ l with Roche LightCycler Taqman master kit, 8 mg/ml BSA, 0.70 pmol of each *D. fragilis* specific primer, 0.25 pmol of each *G. intestinalis* primer, 0.15 pmol of each *Cryptosporidium* sp. primer, 0.5 pmol of each PhHV-1 specific primer, 0.15 pmol of *D. fragilis* specific VIC labelled probe, 0.05 pmol of *G. intestinalis* specific FAM labelled probe, 0.15 pmol of *Cryptosporidium* sp. specific Texas Red labelled probe, 0.75 pmol PhHV-1 specific Cy5 labelled probe and 5 μ l of 1:20 diluted DNA sample. Amplification and detection of protozoan DNA was performed on all samples using the Roche LightCycler 480. Amplification consisted of 10 min at 95 °C followed by 45 cycles of 10 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C. Fluorescence was measured at wavelengths; 483–533 nm (FAM), 523–568 nm (VIC), 558–610 nm (TEXAS RED) and 615–670 nm (CY5) during the annealing step of each cycle.

Amplification of *A. duodenale*, *A. lumbricoides*, *S. stercoralis* and *N. americanus* DNA was performed as a multiplex real time PCR in a volume of 25 μ l with Roche LightCycler Taqman master kit, 8 mg/ml BSA, 0.5 pmol of each *A. duodenale* specific primer, 0.2 pmol of each *A. lumbricoides* primer, 0.25 pmol of each *S. stercoralis* primer, 0.5 pmol of each *N. americanus* specific primer, 0.5 pmol of each PhHV-1 specific primer, 0.25 pmol of *A. duodenale* specific FAM labelled probe, 0.1 pmol of *A. lumbricoides* specific VIC labelled probe, 0.125 pmol of *S. stercoralis* specific ATTO425 labelled probe, 0.125 pmol *N. americanus* specific Texas Red labelled probe,

0.75 pmol PhHV-1 specific Cy5 labelled probe and 5 μ l of 1:20 diluted DNA sample. Amplification conditions were the same as the other real-time PCRs.

2.9. Statistical analysis

Data were entered in Microsoft Excel 2016 and analysed using Stata 13 statistical software. Descriptive statistics were presented for the variables of interest. We assessed differences in the occurrence of each parasite between children (<18 year-olds) and adults (\geq 18 year-olds) and between males and females using multivariable logistic regression models. All analyses included cluster-robust standard errors to account for clustering (or non-independence) of participants living in the households. When some of the cells formed by the outcome and predictor variable had no observations, exact logistic regression was used instead of ordinary logistic regression; correction for within-cluster correlation was maintained. Associations were expressed as odds ratios (OR) and corresponding 95% confidence intervals (95%CI). Chi-square or Fisher's exact tests were used to test the associations between microscopy and PCR positivities. The "gold standard" used was the addition of the positivities for both microscopy and PCR. The degree of agreement between diagnostic techniques was assessed with the kappa (κ) coefficient and corresponding 95%CI. Statistical significance for all statistical tests was set at $p < 0.05$.

3. Results

3.1. Prevalences of helminths

Prevalence rates of intestinal helminths detected using a combination of microscopy and PCR were 49.3% (95%CI 36.7–62.0%) for *A. lumbricoides*, 10.1% (95%CI 4.7–20.5%) for hookworms, and 2.0% (95%CI 0.7–6.0%) for *S. stercoralis*. Additionally, *T. trichiura* and *E. vermicularis* had prevalences of respectively 25.7% (95%CI

Table 2

Prevalence of helminth and protozoan infections using microscopy, PCR and combined techniques in the village “Caserio El 25”, Carabobo state, Venezuela, 2010.

Intestinal parasites		Diagnostic method					
		Only microscopy		Only PCR		Combined microscopy and PCR	
		Positives	Prevalence% (95%CI)*	Positives	Prevalence% (95%CI)*	Positives	Prevalence% (95%CI)*
Helminths (n = 148)	<i>A. lumbricoides</i>	53	35.8 (24.7–48.7) †	63	42.6 (31.3–54.6) †	73	49.3 (36.7–62.0)
	Hookworms**	12	8.1 (3.4–18.1) ‡	13	8.8 (3.8–19.1) ‡	15	10.1 (4.7–20.5)
	<i>S. stercoralis</i>	0	0.0 (0.0–2.5) ‡	3	2.0 (0.7–6.0)	3	2.0 (0.7–6.0)
	<i>T. trichiura</i> ***	38	25.7 (16.3–38.0)	–	–	–	–
	<i>E. vermicularis</i> ***	5	3.4 (1.2–9.2)	–	–	–	–
Pathogenic or potentially pathogenic protozoans (n = 228)	<i>E. histolytica/dispar</i> ****	18	7.9 (4.5–13.4)	0	0.0 (0.0–1.6) ‡	18	7.9 (4.5–13.4)
	<i>D. fragilis</i>	38	16.7 (11.7–23.1) ‡‡	81	35.5 (29.7–41.9) ‡‡	92	40.4 (33.7–47.4)
	<i>G. intestinalis</i>	31	13.6 (9.2–19.7) ‡‡‡	77	33.8 (26.4–42.0) ‡‡‡	80	35.1 (27.4–43.6)
	<i>Cryptosporidium</i> sp.	0	0.0 (0.0–1.6) ‡	0	0.0 (0.0–1.6) ‡	0	0.0 (0.0–1.6) ‡
	<i>Blastocystis</i> sp.***	150	65.8 (58.6–72.3)	–	–	–	–
Non-pathogenic protozoans (n = 228)***	<i>E. coli</i>	66	28.9 (19.8–40.2)	–	–	–	–
	<i>E. hartmanni</i>	28	12.3 (8.1–18.2)	–	–	–	–
	<i>E. nana</i>	45	19.7 (14.6–26.1)	–	–	–	–
	<i>I. bütschlii</i>	17	7.5 (3.6–14.9)	–	–	–	–

95%CI = 95% confidence interval.

* Adjusted for clustering of participants at the household level.

** Only *N. americanus* was detected by PCR. *N. americanus* and *A. duodenale* are not differentiable by microscopic observation of eggs of the parasites.

*** PCR was not available for these helminths or protozoans.

**** PCR available only for *E. histolytica*. Both *E. histolytica* and *E. dispar* are not differentiable by microscopic observation of trophozoites or cysts in normal faeces.

† One-sided, 97.5% confidence interval.

Statistical comparisons between microscopy and PCR were carried out using chi square.

‡ $p < 0.05$, kappa index 0.58 (95%CI 0.44–0.71).‡‡ $p > 0.05$, kappa index 0.78 (95%CI 0.59–0.97).‡‡‡ $p < 0.05$, kappa index 0.29 (95%CI 0.15–0.44).‡‡‡‡ $p < 0.05$, kappa index 0.40 (95%CI 0.26–0.55).

16.3–38.0%) and 3.4% (95%CI 1.2–9.2%) detected only by microscopy since no primers were available (Table 2).

3.2. Prevalences of pathogenic or potentially pathogenic protozoans

Pathogenic or potentially pathogenic protozoan parasites showed prevalences of 35.1% (95%CI 27.4–43.6%) for *G. intestinalis*, 40.4% (95%CI 33.7–47.4%) for *D. fragilis*, and 7.9% (95%CI 4.5–13.4%) for *E. histolytica/dispar*. However, no *E. histolytica* was detected by PCR, and no *Cryptosporidium* sp. was found either by microscopy or PCR. Additionally, *Blastocystis* sp. was found at a prevalence of 65.8% (95%CI 58.6–72.3%) by microscopy only (Table 2).

3.3. Prevalences of non-pathogenic protozoans

The prevalences of non-pathogenic protozoans detected by microscopy only were: *E. coli* 28.9% (95%CI 19.8–40.2%), *E. hartmanni* 12.3% (95%CI 8.1–18.2%), *E. nana* 19.7% (95%CI 14.6–26.1%) and *I. bütschlii* 7.5% (95%CI 3.6–14.9%) (Table 2).

3.4. Prevalence according to age and gender

Prevalences were significantly higher in children than in adults for *A. lumbricoides*, *T. trichiura* and *G. intestinalis*, whereas *E. histolytica/dispar* occurred significantly less often in children than in adults (Table 3). No significant differences in the prevalences of other potentially- or non-pathogenic protozoans were found over age groups (Table 3). Comparisons of prevalences by gender did not show any significant difference for any of the pathogenic and non-pathogenic parasites, with the exception of *E. nana*, for which females were significantly more infected than males (24.2% against 12.4%, $p = 0.039$, not shown on tables).

3.5. Microscopy vs RT-PCR

For helminth infections, the use of RT-PCR in stools resulted in significant higher prevalence for *A. lumbricoides* compared to microscopy. The use of RT-PCR rendered a slightly higher prevalence for hookworms, being all of them *N. americanus*, and exclusively detected the few *S. stercoralis* cases (Table 2). For protozoan infections, RT-PCR for *D. fragilis* and *G. intestinalis* infections doubled significantly the prevalence obtained by microscopy (Table 2). All *E. histolytica/dispar* diagnosed by microscopy could not be detected as *E. histolytica*, according to RT-PCR.

3.6. Diagnostic indexes by microscopy and PCR

The parasites were detected by a combination of microscopy and PCR, therefore, in the majority of cases the patients were double positive, but in some cases they were positives only at microscopy or positives only at PCR. Taking into account the combination: microscopy (+) or PCR (+) as positives, and microscopy (–) and PCR (–) as negatives, the sensitivity of the PCR for detecting *A. lumbricoides* was 86.3%, while for microscopy was 72.6%, for *Necator americanus* the PCR sensitivity was 86.7%, while for microscopy was 85.7%. For *S. stercoralis*, the sensitivity was 100% since all positives were detected by PCR. The PCR sensitivity for detection of *G. intestinalis* was 96.3% while by microscopy was 38.8%. For *D. fragilis*, the PCR sensitivity was 88% while by microscopy it was 41.3%. For *E. histolytica*, the PCR sensitivity was not calculated since it was not detected by PCR (Table 4). Given that the reference group derived from the combination of microscopy and PCR, the specificity and positive predictive values were omitted, as both were always 100%.

The best agreement between microscopy and PCR was for detection of *N. americanus* (Kappa index 0.78) followed by *A. lumbricoides* (Kappa index 0.58), while the lowest agreement was for detection of *D. fragilis* (Kappa index 0.29) followed of *G. intestinalis* (Kappa index 0.40) (Table 2).

Table 3

Parasite prevalence per age group using combined microscopy and PCR in the village “Caserio El 25”, Venezuela, 2010.

Parasite	Children (<18 years)		Adults (≥18 years)		OR (95% CI) [†]	P-value
	n (positives)	% Prevalence (95% CI) [†]	n (positives)	% Prevalence (95% CI) [†]		
Helminths (n = 148)						
<i>A. lumbricoides</i>	83 (49)	59.2 (44.0–74.4)	65 (24)	36.7 (22.7–50.7)	0.40 (0.20–0.79)	0.009
Hookworms	83 (7)	8.1 (0.2–16.1)	65 (8)	12.9 (2.9–22.9)	1.68 (0.54–5.16)	0.368
<i>S. stercoralis</i>	83 (0)	0.0 (0.0–4.3) [‡]	65 (3)	4.6 (1.5–13.1)	1.56 (0.18–∞)	0.370
<i>T. trichiura</i> **	83 (32)	38.5 (23.0–54.0)	65 (6)	9.3 (0.3–18.3)	0.16 (0.05–0.51)	0.002
<i>E. vermicularis</i> **	83 (4)	4.7 (0.0–10.1)	65 (1)	1.6 (0.0–4.7)	0.33 (0.03–3.22)	0.339
Pathogenic or potentially pathogenic protozoans (n = 228)						
<i>E. histolytica/dispar</i> §	122 (6)	4.9 (0.7–9.2)	106 (12)	11.3 (5.3–17.2)	2.44 (1.01–5.89)	0.048
<i>D. fragilis</i>	122 (52)	42.9 (35.4–50.4)	106 (40)	37.4 (28.6–46.3)	0.80 (0.53–1.20)	0.273
<i>G. intestinalis</i>	122 (61)	49.7 (39.2–60.2)	106 (19)	18.1 (10.2–26.0)	0.22 (0.12–0.41)	0.000
<i>Cryptosporidium</i> sp.	122 (0)	0.0 (0.0–3.0) [‡]	106 (0)	0.0 (0.0–3.4) [‡]	n.c.	n.c.
<i>Blastocystis</i> sp.**	122 (77)	63.1 (54.5–71.7)	106 (73)	68.9 (60.5–77.2)	1.29 (0.81–2.05)	0.276
Non-pathogenic protozoans (n = 228)***						
<i>E. coli</i>	122 (35)	29.0 (17.0–40.9)	106 (31)	28.9 (17.9–40.0)	1.00 (0.58–1.73)	0.995
<i>E. hartmanni</i>	122 (12)	9.8 (3.4–16.1)	106 (16)	15.2 (8.2–22.2)	1.66 (0.70–3.90)	0.246
<i>E. nana</i>	122 (19)	15.8 (9.1–22.6)	106 (26)	24.1 (16.0–32.2)	1.71 (0.91–3.21)	0.096
<i>I. bütschlii</i>	122 (10)	8.3 (0.5–16.1)	106 (7)	6.5 (1.9–11.1)	0.77 (0.27–2.24)	0.633

OR = Odds ratio; 95%CI = 95% confidence interval, n.c. = not calculable.

^aAdjusted for age, gender, and clustering of participants at the household level.^{**}Diagnosis made using only microscopy.^{***}PCR was not available for these helminths or protozoans.[§]PCR available only for *E. histolytica*.[†]One-sided, 97.5% confidence interval.

4. Discussion

In this study, we investigated intestinal parasites in a Venezuelan rural community subject to periodic deworming treatments. This was done to improve diagnostic performance and to determine prevalence rates for helminthic and protozoan parasites, which is crucial to calibrate and optimise control campaigns. Real-time PCR and microscopy were used to detect the following pathogenic parasites: *G. intestinalis*, *D. fragilis*, *Cryptosporidium* sp., *E. histolytica*, *A. lumbricoides*, hookworms and *S. stercoralis*. Results showed that RT-PCR gives higher prevalence rates than microscopy for *G. intestinalis*, *D. fragilis*, *A. lumbricoides* and *S. stercoralis*. No *E. histolytica* could be detected by PCR and, consequently, we considered that all *E. histolytica/dispar* detected by microscopy were *E. dispar*. No *Cryptosporidium* sp. was detected either microscopically or by PCR. Other studies have clearly shown the improved sensitivity of PCR over microscopy (Karani et al., 2007; Verweij and van Lieshout, 2011; Stark et al., 2011; Van Lint et al., 2013; Phuphisut et al., 2014; Van den Bossche et al., 2015; Becker et al., 2015; Flecha et al., 2015).

The advantage of using PCR is that it can detect DNA of intact as well as debris of parasites. It can also detect parasites difficult to diagnose like *S. stercoralis* (Basuni et al., 2011; Schär et al., 2013) or those with day to day variation in faecal shedding like *G. intestinalis* (Taniuchi et al., 2011; Stark et al., 2011; Van Lint et al., 2013; Liu et al., 2013; Phuphisut et al., 2014; Van den Bossche et al., 2015; Flecha et al., 2015). The difference between prevalence by microscopy and that by PCR is also highlighted by the microscopical examination of two consecutive faecal samples, i.e. 2 wet mounts plus 2 stained slides per participant. In most cases, the detection of intestinal parasites in stool samples is based on microscopic examination, which is a rather time-consuming analysis. New diagnostic approaches are needed for efficient processing of protozoan parasites. Low grade infections will be missed by microscopy because identification depends on individual experience and skills. The use of a real-time PCR in developing countries is still limited because the costs are much higher than those of microscopic examination (Schoorman et al., 2007), although more recent PCR techniques compete with microscopy in costs (Basuni et al., 2011).

An advantage of microscopy, however, is the broad range of different parasites that can be detected. Using PCR to detect different helminths (eggs or worms) may appear less relevant, as eggs can easily be found by microscopy in unstained wet mounts, particularly from people with moderate or high infection intensity. For low intensity of infection, the Kato-Katz technique is more useful. However, we found a very important increase in prevalence of *A. lumbricoides* using PCR. Although this difference may be partially due to low parasitic loads, the presence of unisexual infections cannot be ruled out. Female worms can produce unfertile eggs, and these can be diagnosed, although sometimes with difficulties, but unisexual infections by male worms are not detected. We have found an important rate of unisexual infections after pirantel treatment and 2 days total worm faecal collection in this community (Inceni, unpublished observations). This community had nearly 5% prevalence of *S. stercoralis* infections using microscopic examination of fixed samples carried out in 2007 (Sequera et al., 2008). Positive patients were treated with ivermectin in 2008, which may be a reason as to why no cases were now detected by microscopy. However, 3 cases were detected by PCR, indicating the higher performance of this technique for cryptic infections.

It is interesting to note that only *N. americanus*, but not *A. duodenale*, was detected in this study. Previous studies worldwide have reported rates of *N. americanus* and *A. duodenale* infections of about 84% and 16%, respectively (Faust et al., 1976; Stoll 1947), and although *N. americanus* is the most prevalent hookworm in the Americas (Faust et al., 1976), no recent figures are available. Now that there are molecular markers for both species, it would be interesting to update studies on the global distribution of both parasites, as well as studies on their phylogeny, which may also help anthropologists to study routes of migration of human populations.

A major drawback of the TFT method is that it is not meant to measure the intensity of STH infections. In contrast, RT-PCR can provide quantitative results. We already quantified the eggs per gram of faeces in this community and showed (using the MIF method) that the percentages of people with moderate to high infection intensity were 54.5%, 62.5% and 47.1% for *T. trichiura*, *A. lumbricoides* and hookworms, respectively, further indicating that

Table 4
Diagnosis indexes of microscopy and PCR for evaluated helminths and protozoans.

Microscopy	Sensitivity (%)	NPV** (%)
<i>Ascaris lumbricoides</i>	72.6	78.9
Hookworms	85.7	98.5
<i>Necator americanus</i>	–	–
<i>Ancylostoma duodenale</i>	–	–
<i>Strongyloides stercoralis</i>	–	–
<i>Entamoeba histolytica/dispar</i>	100	100
<i>Entamoeba histolytica</i>	–	–
<i>Dientamoeba fragilis</i>	41.3	73.3
<i>Giardia lamblia</i>	38.8	75.1
<i>Cryptosporidium</i> sp.	–	–
PCR		
<i>Ascaris lumbricoides</i>	86.3	88.2
Hookworms	–	–
<i>Necator americanus</i>	86.7	98.5
<i>Ancylostoma duodenale</i>	–	–
<i>Strongyloides stercoralis</i>	100	100
<i>Entamoeba histolytica/dispar</i>	–	–
<i>Entamoeba histolytica</i>	–	–
<i>Dientamoeba fragilis</i>	88.0	93.1
<i>Giardia lamblia</i>	96.3	98.0
<i>Cryptosporidium</i> sp.	–	–

The reference group resulted from the positivities for both microscopy and PCR. Consequently, specificity and positive predictive values are all 100%.

* PPV Positive Predictive value.

** NPV Negative Predictive value.

transmission of STH is relevant in the community (Incani et al., 2016).

G. intestinalis is the one of the most prevalent pathogenic protozoan infections worldwide, with currently over 200 million infected people (Esch and Petersen 2013). High prevalence of *G. intestinalis* was also found in this study using both microscopy and PCR. However, PCR highly increased the detection rate indicating the higher sensitivity of this technique for a parasite with irregular shedding. The number of *D. fragilis* infections is commonly underestimated. The use of *D. fragilis* PCR is necessary because microscopic diagnosis is hindered by its quick decomposition (it has no cyst stage, only trophozoites) (Yang and Scholten 1977; Stensvold and Nielsen 2012) and additionally, it requires more technical expertise. Microscopic detection also relies on fresh stool samples that are fixed immediately after collection. PCR turned out to be more sensitive in detecting *D. fragilis*.

Cryptosporidium sp. was not found using microscopy, nor using PCR. This is unusual for a community with high faecal contamination given the other intestinal parasite prevalences we found. Studies worldwide have shown different prevalences in asymptomatic people varying, for example, from 0.4% in a community-based study in Australia, 1.3% in day-care centres in the UK, to 31.6% in Aymara indigenous populations of Bolivia (Reviewed by Cacció and Putignami, 2014). Our negative results could be explained by seasonal variation which appears to be frequent for this parasite. However, a total negative result for PCR diagnosis is still intriguing and we may think on possible genetic variability of the circulating strains in the country so that it could be necessary to use strain-specific primers or degenerate primers for the PCR.

E. histolytica is together with *G. intestinalis* the most common waterborne pathogenic intestinal protozoan (Esrey et al., 1985; Steiner et al., 1997; Ashbolt 2004; Mtapuri-Zinyowera et al., 2009). Considering that *E. histolytica* and *E. dispar* cannot be differentiated morphologically, the 18 cases (all asymptomatic, data not shown) detected by microscopy were reported as *E. histolytica/dispar*. However, none were found using *E. histolytica* primers. This emphasises the need to better diagnose this parasite species, because many infected people are being treated purposeless.

The age distribution of the parasite prevalences in this community warrants some comments. Individuals below 18 years of age were more likely to be infected with *A. lumbricoides*, *T. trichiura* and *G. intestinalis*, but not with the others. For helminths, it is generally acknowledged that before puberty (below 15 years of age), the prevalence, but mostly the intensity of infection, peaks for *A. lumbricoides* and *T. trichiura*. However, for hookworms, the prevalence is higher during young adulthood (Hotez et al., 2006, 2008), probably reflecting age-related resistance. For protozoan infections, there is a paucity of information concerning their age-related distribution in the population, and most works are related to infection in children or clinical cases. The US Center for Disease Control (CDC) produced figures showing a peak in *G. intestinalis* prevalence at the age of 1–4 years during 2011–2012 (Painter et al., 2015). Age-related studies on *E. histolytica* are hampered by the necessity to differentiate it from *E. dispar* using other methods than morphology. However, the early population study of Rivera et al. (1998) in the Philippines evidenced that the combined prevalence of *E. histolytica* and *E. dispar*, diagnosed by PCR, is significantly higher at the age group of 5–14 years as compared to younger (1–4 years) or older (>45 years) groups. There seems to be no consensus on the age-related prevalences for *D. fragilis* (Barratt et al., 2011) or *Blasotocystis* sp. (Belleza et al., 2015). Non-pathogenic protozoans did not show significant age-related differences in prevalence. Although this has to be confirmed in other settings and with higher number of observations, we may think that the absence of age-related prevalences of commensal protozoans is consistent with their likely role as part of the intestinal microbiota, being possibly unaffected by limiting factors like age-related development of resistance in conditions of low sanitization like those present in this community.

In conclusion, we have shown that diagnosis of intestinal parasites based on RT-PCR has a good performance under field conditions, providing prevalence rates for most of the parasites studied, which will be otherwise under-estimated. Designing multiplex PCRs that include all pathogenic intestinal parasites, helminths and protozoan, should be sought in order to avoid underestimation of prevalences. It also helps to better understand the influence of intestinal parasitic infections and the situation of dual burden households found in communities like this one (Campos-Ponce et al., 2012). However, we must stress the importance of combining molecular methods with microscopy, as even if all pathogens can be detected using a multiplex approach, PCR remains an expensive technique for low-resource settings. Moreover, it does not yet include non-pathogenic protozoans as part of the microbiota, which are increasingly being recognized as key players in health and disease (Maranduba et al., 2015; Giacomini et al., 2015).

Conflict of interests

The authors declare no conflicts of interest and that they are responsible for the content and writing of this article.

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