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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 anthelminthic 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 monoplex 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.

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 hookworms 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 (Hashmey 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-fuchsine (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 1 x PBS. After centrifugation, the pellet was resuspended into 200 μl of freshly prepared lysis buffer (2% polyvinylpolypyrrolidone, 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