

Parasitology

# Real-time polymerase chain reaction for detection of *Isoospora belli* in stool samples

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## Abstract

A real-time polymerase chain reaction (PCR) assay targeting the internal transcribed spacer 2 region of the ribosomal RNA gene was developed for the detection of *Isoospora belli* DNA in fecal samples, including an internal control to detect inhibition during the amplification process. The assay was performed on species-specific DNA controls ( $n = 27$ ) and a range of positive ( $n = 21$ ) and negative ( $n = 120$ ) stool samples, and achieved 100% specificity and sensitivity. The simple fecal sample collection procedure, the high-throughput potential, and the possibility of quantification makes the *I. belli* real-time PCR assay a powerful diagnostic tool for epidemiologic studies with possibilities for extension to other helminthes and protozoa using additional molecular targets. In addition, this *Isoospora* PCR could augment the clinical laboratory diagnosis of isosporiasis, in particular, in patients with a travel history to developing countries.

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## 1. Introduction

Infection with the intestinal protozoa *Isoospora belli* is associated with chronic and severe diarrhea, in particular, for persons living with AIDS and other immunocompromised individuals (Atambay et al., 2007; Ferreira, 2000; Lewthwaite et al., 2005). Infections are also seen in children and travelers to tropical regions (Goodgame, 2003; Jongwutiwes et al., 2007; Okhuysen, 2001). Although symptoms are self-limiting in immunocompetent individuals, early diagnosis and treatment can shorten the period of intestinal symptoms substantially. Diagnosis is usually made by microscopic detection of the parasite oocysts in stool samples. The oocysts have a thin transparent shell that makes detection of the oocysts in unstained direct smears difficult. To improve sensitivity,

we have to perform additional microscopic, concentration, and/or staining methods (Bialek et al., 2002; Franzen et al., 1996; Lainson and da Silva, 1999).

A nested polymerase chain reaction (PCR) method with Southern blot hybridization was described by Muller et al. (2000) as a helpful technique for the detection of very mild *I. belli* infections. However, these are very laborious procedures and, therefore, not efficient for analyzing large number of samples. Recent studies have demonstrated the detection of parasite DNA in feces with real-time PCR to be a sensitive and specific alternative technique for the diagnosis of intestinal parasitic infections (ten Hove et al., 2007; Verweij et al., 2007). Real-time PCR is a closed system that can provide quantitative information for up to 3 targets, including an internal control in 1 multiplex assay.

In the present study, a real-time PCR was developed for the specific detection of *I. belli* DNA in fecal samples. In addition, an internal control for the detection of possible inhibition of the amplification by fecal contaminants was included in the assay. The performance of the PCR was evaluated using a large range of DNA controls.

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## 2. Materials and methods

### 2.1. Controls and samples

To establish the real-time PCR assay, we used *I. belli* control DNA extracted from an unpreserved human fecal sample in which the presence of *I. belli* oocysts was confirmed by microscopy.

Specificity of the PCR assay was evaluated using 1) DNA isolated from individual adult worms of *Schistosoma mansoni* and *Necator americanus* and also DNA of *Strongyloides stercoralis* 1st-stage larvae and *Ancylostoma duodenale* 3rd-stage larvae, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, *Cryptosporidium parvum*, *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* (Verweij et al., 2007), *Dientamoeba fragilis* DNA isolated from culture (ATCC 30948), and *Toxoplasma gondii* DNA isolated from RH reference strain of *T. gondii* maintained in female Swiss Webster mice by intraperitoneal passage; 2) DNA obtained from different bacterial/yeast cultures of *Enterococcus faecalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Enterobacter aerogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, *Proteus mirabilis*, *Candida albicans*, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei*, and *Escherichia coli* O157; and 3) 80 DNA extracts from unpreserved stool samples from different patients in which *E. histolytica* ( $n = 20$ ), *E. dispar* ( $n = 20$ ), *G. lamblia* ( $n = 20$ ), or *C. parvum*/*Cryptosporidium hominis* ( $n = 20$ ) were detected by microscopy and confirmed by specific PCR, respectively (Verweij et al., 2003b, 2004). The specificity of the PCR assay was also tested on DNA extracts from 40 unpreserved stool samples with a negative result in microscopy using formalin–ether sediments and modified acid-fast staining, and a *Giardia* antigen test. For these negative samples, 2 subsequent stool samples from these patients tested negative by microscopy and *Giardia* antigen detection.

The PCR was further evaluated using DNA extracts of 21 stool samples from individual patients in which the microscopic examination of modified acid-fast stained fecal smears revealed *I. belli* oocysts. Eighteen stool samples originated from the Queen Elizabeth Central Hospital in Blantyre, Malawi, where HIV/AIDS is highly endemic. Three stool samples were used from travelers to Nigeria, India, and Nepal, diagnosed at the Institute of Tropical Medicine in Antwerp, Belgium ( $n = 2$ ), and the Albert Sweitzer Hospital in Dordrecht, The Netherlands, respectively.

Furthermore, DNA was isolated from serial fecal dilution series with 100 oocysts to estimate the sensitivity of the PCR.

### 2.2. DNA isolation

For DNA isolation, approximately 100 mg of unpreserved feces were suspended into 200  $\mu$ L of phosphate-buffered saline containing 2% polyvinylpyrrolidone (Sigma,

Steinheim, Germany). After heating for 10 min at 100 °C, suspensions were treated with sodium dodecyl sulfate–proteinase K for 2 h at 55 °C. DNA was then isolated using QIAamp Tissue Kit spin columns (QIAGEN, Hilden, Germany) (Verweij et al., 2001). If formalin-fixed fecal samples are used, the formalin has to be removed by washing/centrifuging steps with saline direct or after formalin–ether concentration. However, sensitivity of amplification is known to decrease with time of fixation (Ramos et al., 1999). Within the isolation lysis buffer, 10<sup>3</sup> plaque-forming units (PFU)/mL phocin herpes virus 1 (PhHV-1) was added to serve as an internal control (Niesters, 2002).

### 2.3. PCR amplification and detection

*I. belli*-specific primers and detection probe were designed using Primer Express software (Applied Biosystems, Foster City, CA) on the internal transcribed spacer 2 ribosomal RNA gene sequence (GenBank accession no. AF443614). For detection of *I. belli*, primers Ib-40F (5'-ATA TTC CCT GCA GCA TGT CTG TTT-3') and Ib-129R (5'-CCA CAC GCG TAT TCC AGA GA-3') were selected to amplify a fragment of 89 bp, which was detected by the double-labeled probe Ib-81Taq (FAM-5'-CAA GTT CTG CTC ACG CGC TTC TGG-3'-BHC1) (Biolegio, Malden, The Netherlands). For simultaneous detection of internal control, PhHV-1-specific primers and probe set consisted of forward primer PhHV-267s, reverse primer PhHV-337as, and the specific Cy5-BHQ2-labeled probe PhHV-305tq (Biolegio) (Niesters, 2002). National Center for Biotechnology Information (NCBI) BLAST search was used to test the theoretical specificity of the primers and probe.

A 10-fold dilution series of *I. belli* control DNA was used to optimize the real-time PCR and tested with and without the presence of internal control DNA to estimate the latter's influence.

For DNA amplification, 5  $\mu$ L of DNA extracted from stool specimens was used as a template in a final volume of 25  $\mu$ L with 2 $\times$  PCR buffer (HotstarTaq master mix, QIAGEN), 5 mmol/L MgCl<sub>2</sub>, 2.5  $\mu$ g bovine serum albumin (Roche Diagnostics Nederland, Almere, The Netherlands), 60 nmol/L of each *I. belli*-specific primer, 150 nmol/L of each PhHV-1-specific primer, and 100 nmol/L of *I. belli*- and PhHV-1-specific double-labeled probes. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification, detection, and data analysis were performed with the Applied Biosystems 7500 Real-Time PCR system and Sequence Detection Software version 1.2.2.

## 3. Results

In the NCBI BLAST search, primers and probe showed 100% specificity for *I. belli*.

The real-time PCR was optimized using a 10-fold dilution series of *I. belli*-positive control DNA. The cycle

threshold (Ct) values obtained from testing the dilution series of *I. belli* DNA in both the individual *I. belli* assay and the multiplex assay with the internal control showed no systemic deviation in amplification curves, and the same analytical sensitivity was achieved. The individual performance of the assays was not influenced by the presence of DNA from the internal control.

The specificity of the real-time multiplex PCR was evaluated using a range of parasite and bacterial control DNAs ( $n = 27$ ), 80 DNA extracts derived from feces positive for *E. histolytica*, *E. dispar*, *G. lamblia*, or *C. parvum/C. hominis*, and 40 DNA extracts derived from feces of individuals with no known history of parasitic infections. No amplification of *I. belli*-specific DNA was detected in any of these 147 samples; only the amplification of the internal control was detected at the expected threshold cycle of approximately 30.

*I. belli*-specific amplification was detected in all 21 samples in which *I. belli* was detected by microscopic examination, with Ct values of between 22.8 and 30.5 with a median threshold of 25.8 cycles. One *I. belli* oocyst was estimated as the detection limit of the isolation and PCR procedure. Fecal suspensions containing 100-, 10-, or 1 oocyst corresponded with Ct values 30.0, 33.2, and 36.3, respectively.

#### 4. Discussion

Among immunocompromised individuals, especially those living with AIDS, the most important opportunistic intestinal parasites include *Cryptosporidium* spp., microsporidia, and *I. belli*. Additional staining techniques are required for microscopic detection of all these organisms. Nevertheless, light infections can still be easily missed. Already, highly sensitive and specific real-time PCR procedures have been validated for the detection of *Cryptosporidium* spp. (ten Hove et al., 2007; Verweij et al., 2004), *E. bieneusi*, and *Encephalitozoon* spp. (Verweij et al., 2007).

In the present study, an *I. belli* real-time PCR was developed to complete the series of molecular diagnostic assays for the detection of opportunistic intestinal parasites. Using a large range of control DNA and stool samples, the real-time PCR achieved 100% specificity. In a selected group of 21 fecal samples from patients in which *I. belli* was shown by microscopy, the real-time PCR showed a sensitivity of 100%. Considering the low Ct values obtained in these microscopy-positive samples and the detection limit of 1 oocyst in a fecal suspension, this real-time PCR has the potential to detect *I. belli* infections in patients shedding very low numbers of oocysts. A prospective study on the sensitivity of the real-time PCR as compared by microscopic techniques is planned for the near future.

In Western countries, isosporiasis is diagnosed occasionally, and those with higher risk are travelers and immigrants. Nevertheless, the true number of cases could be underestimated, and more cases are probably detected in routine

screening of specific patient groups with real-time PCR for *I. belli*. This real-time PCR could be part of routine posttravel screening multiplex PCR extended with other targets such as *C. cayetanensis* (Verweij et al., 2003a).

Obviously, this real-time PCR will not be appropriate as a routine diagnostic tool in clinical settings in endemic areas where resources of the laboratories are often limited. However, the high-throughput potential and species-specific quantification as a measure of intensity of infection can be used, together with other collected health and demographic indicators, as a powerful tool for epidemiologic studies or to monitor effectiveness of treatment in *I. belli* infections (Pape et al., 1989; Verdier et al., 2000). Stool samples mixed with ethanol allow the samples to be stored and transported at room temperature to laboratories with the appropriate facilities for DNA extraction and detection. Moreover, once DNA is extracted, the real-time PCR assay can be extended to other helminths or protozoa using additional molecular targets. In addition, up to 4 molecular targets with different fluorescent labels can be combined as a multiplex real-time PCR assay, leading to considerable reduction in reagent costs.

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