Journal of Medical Microbiology (2003), **52**, 681–683 **DOI** 10.1099/jmm.0.05193-0

Short Communication

Detection and genotyping by real-time PCR/RFLP analyses of *Giardia duodenalis* from human faeces

Corinne F. L. Amar,[1] Paul H. Dear[2] and Jim McLauchlin[1]

[1]Health Protection Agency, Food Safety Microbiology Laboratory, Division of Gastrointestinal Infections, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, UK

[2]Medical Research Council, Laboratory of Molecular Biology, Cambridge CB2 2QH, UK

Correspondence
Jim McLauchlin
jim.mclauchlin@hpa.org.uk

Received 24 January 2003
Accepted 15 April 2003

A nested PCR assay (TPILC-PCR) was developed to detect and distinguish between *Giardia duodenalis* assemblages A and B from human faeces by analysis of the triose phosphate isomerase gene (*tpi*). The assay comprised an initial multiplexed block-based amplification. This was followed by two separate real-time PCR assays specific for assemblages A and B using a LightCycler and SYBR Green I to identify PCR products by melting-point analysis. RFLP analysis was applied to distinguish *G. duodenalis* assemblage A groups I and II. The real-time nested PCR was evaluated using DNA extracted from purified giardial trophozoites, *Cryptosporidium* oocysts, whole faeces containing a range of potential pathogens (including *G. duodenalis*), faecal smears and bacterial suspensions. The assay was specific, sensitive, reproducible and rapid.

Introduction

In humans, food-borne and water-borne giardiasis due to the protozoan *Giardia duodenalis* (synonym of *Giardia intestinalis* and *Giardia lamblia*) is a common cause of gastroenteritis and a major health concern worldwide (Farthing, 1995). Current methods for detection of this parasite from faeces are usually based on light microscopy (Isaac-Renton, 1991). However, these methods are unable to distinguish between genetically distinct parasites (Isaac-Renton, 1991; LeChevallier *et al.*, 1995). We previously described a sensitive hemi-nested PCR that can detect and genotype *G. duodenalis* assemblages A and B (the two assemblages known to infect humans) from whole faeces and also from faecal smears (Amar *et al.*, 2002). The aim of this study was the further development and evaluation of a simple, highly specific and sensitive real-time PCR technique applicable to whole faeces and stained faecal smears that is able to detect and genotype *G. duodenalis* assemblages A and B.

Methods

Faecal specimens. Faeces that contained *G. duodenalis* assemblage A group II (eight samples); *G. duodenalis* assemblage B (14 samples); *Cryptosporidium parvum* genotypes 1 and 2 (four samples) and *Cyclospora* sp. (two samples) were collected from naturally infected humans (McLauchlin *et al.*, 2000; Amar *et al.*, 2002). All samples were stored at 4 °C without preservatives.

DNA samples. DNA extracts containing 1 ng DNA μl^{-1} from cultured *G. duodenalis* trophozoites of reference strains VNB3 and AMC13 (assemblage A), AMC9 (assemblage B), 265KA1184 (hoofed farm animal) and DOG1 (dog type) were provided by W. Homan (Laboratory for Parasitology and Mycology, Bilthoven, The Netherlands) (Homan *et al.*, 1998). DNA was also extracted from purified oocysts of *Cryptosporidium parvum* genotype 1 (MRC, Laboratory of Molecular Biology, Cambridge, UK), *Cryptosporidium parvum* genotype 2 (Iowa strain 1372; AIDS Research Reference Reagent Program, National Institutes of Health, USA), *Cryptosporidium baileyi*, *Cryptosporidium muris* and *Eimeria tenella* (Patel *et al.*, 1999) and from *in vitro*-grown *Escherichia coli* strain N211 (PHLS Food External Quality Assessment Scheme, London, UK) and *Clostridium perfringens* type A NCTC 8239.

Microscopy. Smears were produced from faecal samples (Amar *et al.*, 2001) and examined by indirect immunofluorescence microscopy as described previously (Amar *et al.*, 2002).

DNA extraction and polyvinyl pyrrolidone (PVP) treatment. DNA extraction from whole faeces and from stained smears on glass microscope slides, including further DNA purification using PVP, was performed as described before (McLauchlin *et al.*, 1999; Amar *et al.*, 2001). DNA from purified oocysts and bacterial suspension was also prepared by a similar method.

Nested real-time PCR amplification for identification of *G. duodenalis* assemblages A and B (TPILC-PCR). The *tpi* gene sequences of *G. duodenalis* assemblage A groups I and II (GenBank accession nos L02120 and U57897, respectively) and assemblage B (L02116 and AF069561) were aligned using the program BioEdit (Hall, 1999). Two sets of four primers (Table 1) were designed to amplify *G. duodenalis* assemblages A and B.

Amplification was performed in two phases. A duplex phase-I PCR was performed using a conventional thermocycler (Biometra T3; Anachem) and primers designed to amplify fragments of the *tpi* gene of *G. duodenalis* of 576 bp from assemblage A (primers TPIA4F/TPIA4R) and 210 bp from assemblage B (primers TPIB4F/TPIB4R) (Table 1). The duplex reaction was performed in a 10 μl volume with 5 μl DNA in 1× PCR buffer, 2 mM $MgCl_2$, 0·25 mM of each dNTP, 0·3 μM of each primer and 0·5 U *Taq* DNA polymerase (all reagents from Invitrogen).

Samples were subjected to an initial denaturation of 94 °C for 1 min, 25 cycles of 94 °C for 20 s, 50 °C for 30 s and 72 °C for 1 min and a final extension at 72 °C for 5 min.

Two separate phase-II PCRs, with inner forward (IF) and reverse (IR) primers, were devised to amplify fragments of the *G. duodenalis tpi* gene of 452 bp from assemblage A (primers TPIA4IF/TPIA4IR) and 141 bp from assemblage B (primers TPIB4IF/TPIB4IR). Both phase-II reactions were performed as real-time hot-start PCRs using a LightCycler (Roche Molecular Biochemicals). The reaction comprised 10 µl of the phase I duplex-PCR product diluted 10 times in nuclease-free water (Sigma), 2 mM $MgCl_2$, 1 µM of each primer (IF/IR) and 2 µl Master Mix (FastStart DNA Master SYBR Green I kit; Roche Molecular Biochemicals) in a volume of 20 µl. Cycling conditions were 95 °C for 8 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 3 s and 72 °C for 10 s, with a transition rate of 20 °C s^{-1}. Fluorescence readings were taken after each extension step and as a final melting analysis by treatment at 95 °C for 0 s, 68 °C for 15 s followed by a transition at 0·1 °C s^{-1} to 95 °C. Melting temperatures (T_m) were derived from melting peaks using LightCycler software version 3.5. Each test batch contained a maximum of 30 samples plus one positive control (AMC13- or AMC9-derived DNA) and one negative control (water).

Gel electrophoresis and RFLP. *Rsa*I restriction sites were identified from an alignment of the *tpi* gene of *G. duodenalis* assemblage A to distinguish between subgenotypes groups I and II. The predicted restriction fragments were 437 and 15 bp for group I and 235, 202 and 15 bp for group II.

PCR products were recovered from LightCycler glass capillaries by centrifugation and RFLP analysis was performed by digesting 5 µl PCR product with 5 U restriction enzyme in 1× enzyme buffer (Invitrogen) in a final volume of 30 µl for at least 4 h at 37 °C. Restriction fragments were separated in 3·2 % agarose/ethidium bromide gels by horizontal electrophoresis and examined by UV transillumination.

DNA sequencing. PCR products were purified using a StrataPrep PCR purification kit (Stratagene). Sequencing of PCR products (sense and antisense) was performed at the Advanced Biotechnology Centre, Imperial College, London, UK, using an ABI 377 automated DNA sequencer and appropriate IF and IR primers.

Table 1. Primers used for amplification of fragments of the *G. duodenalis tpi* gene by TPILC-PCR

Primer positions refer to sequences for *G. duodenalis* assemblages A and B respectively obtained from GenBank accession numbers L02120 and L02116.

Primer	Sequence	Position
Assemblage A		
TPIA4F	5′-CGAGACAAGTGTTGAGATGC-3′	758–777
TPIA4IF	5′-CCAAGAAGGCTAAGCGTGC-3′	859–877
TPIA4IR	5′-GCCACATGCCTATGTACGGG-3′	1306–1318
TPIA4R	5′-GGTCAAGAGCTTACAACACG-3′	1334–1353
Assemblage B		
TPIB4F	5′-GTTGCTCCCTCCTTTGTGC-3′	663–681
TPIB4IF	5′-GCACAGAACGTGTATCTGG-3′	732–750
TPIB4IR	5′-CTCTGCTCATTGGTCTCGC-3′	871–889
TPIB4R	5′-GGCCTTGCGTTCATCCAGG-3′	935–953

Results and Discussion

DNA from all faeces, cyst, oocyst and bacterial suspensions was subjected to the TPILC-PCR/RFLP. Following TPIALC-PCR, melting peaks with a T_m of 90·63–91·74 °C (SD 1·0–1·2) were generated exclusively from DNA recovered from *G. duodenalis* strains VNB3 and AMC13 and eight faecal samples containing *G. duodenalis* assemblage A. RFLP results identified VNB3 as assemblage A group I and AMC13 plus all eight faecal samples as containing assemblage A group II. Following TPIBLC-PCR, melting peaks with a T_m of 87·80–88·44 °C (SD 1·1–1·3) were generated exclusively from DNA recovered from *G. duodenalis* strains AMC9 and VNB3 and 14 faecal samples containing *G. duodenalis* assemblage B. For both phase-II reactions, non-specific PCR products generated either no peaks or flatter peaks with low T_m values (Fig. 1). The sequence of the TPIALC-PCR product from VNB3 was 100 % identical to the *G. duodenalis* assemblage A group I sequence (L02120). Analysis of the products amplified from reference strain AMC13 and one faecal sample showed 100 % sequence identity to the *G. duodenalis* assemblage A group II sequence (U57897) and was therefore consistent with the results from RFLP analysis. TPIBLC-PCR products from AMC9 and VNB3 and one of the faecal samples showed

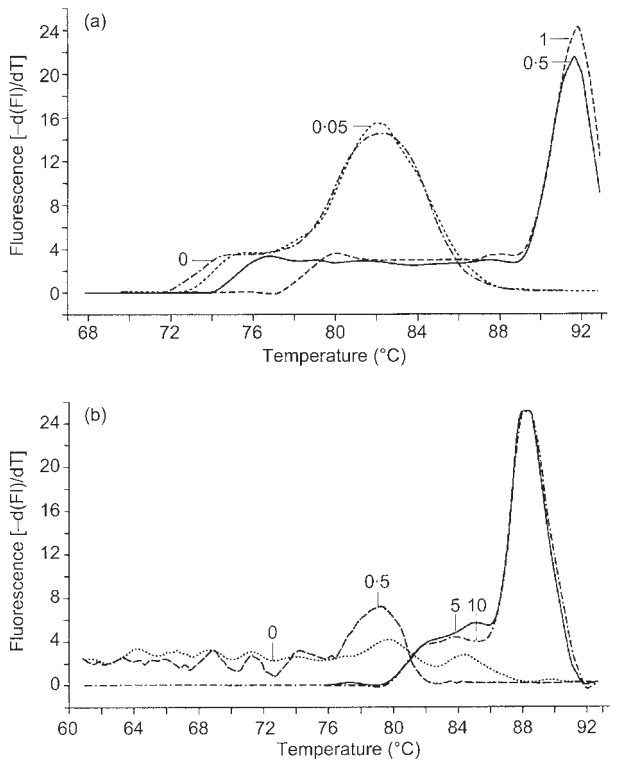

Fig. 1. Sensitivity of real-time TPIALC-PCR (a) and TPIBLC-PCR (b) assays. The graphs show melting-curve analysis for products of amplification from samples containing various amounts of DNA per reaction (indicated for each curve in genome copy equivalents) from reference strains AMC13 (*G. duodenalis* assemblage A) (a) and AMC9 (*G. duodenalis* assemblage B) (b). Specific products have T_m values of approximately 91 °C (a) or 88 °C (b).

sequences identical to the *G. duodenalis* assemblage B sequence (L02116 and AF069561). Sequencing analyses confirmed that the VNB3 DNA extract contained both assemblages A group I and B, and also confirmed the specificity of the PCR assays.

To estimate the limit of detection of the TPILC-PCR, DNA extracts of reference strains were serially diluted in sterile distilled water. The dilutions were subjected to TPIALC (AMC13) or TPIBLC (AMC9) PCR. Specific melting peaks could be observed (Fig. 1) when generated from the TPIALC and TPIBLC PCR amplifications using 0·005 and 0·05 pg of DNA per reaction, respectively, corresponding to 0·5 and 5 copies of the *tpi* gene, based on a genome size of $1{\cdot}2 \times 10^7$ bp (Adam, 2000). The reproducibility of detection of *G. duodenalis* assemblages A and B using DNA extracted from purified AMC13 and AMC9 strains analysed five times by TPILC-PCR was 100 %.

Smears were produced from 20 faecal samples (described above) from which *G. duodenalis* assemblage A group II (seven samples) and assemblage B (13 samples) had previously been detected. All smears were stained by immunofluorescence and giardial cysts were confirmed in all samples: in eight samples, five or fewer cysts were detected per microscope field. DNA was extracted from all of the smears and analysed in triplicate by TPILC-PCR/RFLP. *tpi* gene fragments were amplified from 15 (75 %) of the samples and the assemblages recovered were the same as those previously detected from faeces. Of the 15 smears where the *tpi* fragment was amplified, 10 were positive in all three replicates, four in two replicates and one in one of the three replicates. There was no correlation between reproducibility in triplicate tests and the number of cysts detected by microscopy (data not shown). The number of cysts seen by microscopy may not be proportional to the amount of intact template DNA, since the contents of cysts may be degraded prior to extraction. Therefore, the reduced reproducibility was most likely due to sampling error because of the very low original template concentration.

Phase I of the TPILC-PCR was performed in a conventional thermocycler, and only the nested phase was adapted to the LightCycler system. This format retained the high specificity and sensitivity provided by a nested reaction, and the use of diluted phase-I PCR product avoided saturation of the fluorescence signal by double-stranded DNA recovered from faeces. The sensitivity of the fully nested reaction (0·5–5 copies of *tpi*) was similar to that described previously for a hemi-nested protocol using the same target (Amar *et al.*, 2002). However, this LightCycler assay has a considerable advantage over the previously reported 'block-based' procedure (Amar *et al.*, 2002) because of the speed of analysis. Excluding the RFLP analysis (which is identical for both procedures), the conventional hemi-nested TPI-PCR (Amar *et al.*, 2002) took approximately 3 h and 15 min to perform, compared with 1 h and 50 min for the LightCycler assay described here. However, one disadvantage of using the LightCycler was that each batch was limited to 30 assays plus one positive and one negative control. The use of hybridization probes as a replacement for the RFLP analysis is currently being evaluated, which would further reduce the time required to perform these assays.

Acknowledgements

We thank colleagues in clinical microbiology laboratories for the donation of specimens and Dr W. Homan (Laboratory for Parasitology and Mycology, Bilthoven, The Netherlands) for purified giardial DNA. C. F. L. A. is funded by a PHLS PhD studentship.

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