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 gastro-enteritis 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, 1995). Current methods for detection of this parasite from faeces are usually based on light microscopy (Isaac-Renton, 1995). However, these methods are unable to distinguish between genetically distinct parasites (Isaac-Renton, 1991; LeChevallier et al., 1995). We previously described a sensitive, highly specific and sensitive real-time PCR technique applicable to whole faeces and also from faecal smears on glass microscope slides, including further DNA purification using PVP, 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 suspensions was also prepared by a similar method.

Methods

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

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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.

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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 TPIAIF/TPIAIR) and 141 bp from assemblage B (primers TPIBIF/TPIBIR). 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 MgCl₂, 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 (Tm) 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.** Real 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 bp for group II. Restriction fragments were separated in 3·2 % agarose/ethidium bromide gels by horizontal electrophoresis and examined by UV transillumination.

**DNA sequencing.** PCR products were recovered from LightCycler glass capillaries by centrifugation and RFLP analysis was performed by digesting 5 μl PCR products with 5 μl restriction enzyme in 1% enzyme buffer (Invitrogen) in a final volume of 20 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.

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assemblage A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPIAIF</td>
<td>5'-CGAGACAAATGGTGATGATGC-3'</td>
<td>758–777</td>
</tr>
<tr>
<td>TPIAIF</td>
<td>5'-CCAAAGAGGGCTAAGGTCG-3'</td>
<td>859–877</td>
</tr>
<tr>
<td>TPIAIF</td>
<td>5'-GCCCATGCTGGCTAGTACGG-3'</td>
<td>1306–1318</td>
</tr>
<tr>
<td>TPIAIF</td>
<td>5'-GGTCAAGAGCTTCTACACG-3'</td>
<td>1334–1353</td>
</tr>
<tr>
<td><strong>Assemblage B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPIBIF</td>
<td>5'-GTGTCCTCCTCTCTTTGTCG-3'</td>
<td>663–681</td>
</tr>
<tr>
<td>TPIBIF</td>
<td>5'-GACCAGAAGGTAGTACCTGG-3'</td>
<td>732–750</td>
</tr>
<tr>
<td>TPIBIF</td>
<td>5'-CTGCTGCACTGGCTCCCG-3'</td>
<td>871–889</td>
</tr>
<tr>
<td>TPIBIF</td>
<td>5'-GGCTTGGTTCATCAGG-3'</td>
<td>935–953</td>
</tr>
</tbody>
</table>

**Results and Discussion**

DNA from all faeces, cyst, oocyst and bacterial suspensions was subjected to the TPLLC-PCR/RFLP. Following TPLLC-PCR, melting peaks with a Tm of 90·63–91·74 °C (sd 1·40–1·12) 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 Tm 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 Tm values (Fig. 1). The sequence of the TPLLC-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 TPLLC-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 Tm values of approximately 91 °C (a) or 88 °C (b).](image-url)
sequences identical to the \textit{G. duodenalis} assemblage B sequence (L02116 and AF69561). 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 \textit{tpi} gene, based on a genome size of $1.2 \times 10^7$ bp (Adam, 2000). The reproducibility of detection of \textit{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 \textit{G. duodenalis} assemblage A group II (seven samples) and assemblage B (13 samples) had previously been detected. All smears were stained by immuno-fluorescence 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. \textit{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 \textit{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 \textit{tpi}) was similar to that described previously for a semi-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 semi-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

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References


