**Giardia duodenalis** typing from stools: a comparison of three approaches to extracting DNA, and validation of a probe-based real-time PCR typing assay

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A weighted, multi-attribute approach was used to compare three methods for direct extraction of *Giardia duodenalis* DNA from 15 microscopy-positive stools: (1) a QIAamp spin-column method for stools including a 10 min incubation at 95 °C, (2) method 1 preceded by five freeze–thaw cycles and (3) bead beating with guanidine thiocyanate using a FastPrep-28 machine followed by liquid-phase silica purification of DNA. The attributes compared included DNA yield measured using a new triose phosphate isomerase (*tpi*) gene probe-based real-time PCR, also described here. All three methods shared 100% PCR positivity, while the bead-beating method provided the highest *G. duodenalis* DNA yield (P<0.01). However, when other weighted attributes, including biocontainment, resources and technical requirements, were also considered, spin-column extraction with prior freeze–thaw treatment (method 2) was deemed the most desirable and was selected for use. The *tpi* real-time PCR typing assay was designed to discriminate between the main human infectious assemblages of *G. duodenalis* (A and B) and was evaluated initially using standard isolates. Validation using microscopy-positive stools from 78 clinical giardiasis cases revealed 100% typability; 20 (26%) samples contained assemblage A, 56 (72%) assemblage B and two (3%) assemblages A and B. While the epidemiological significance of assemblage distribution will be revealed as more isolates are typed and analysed with patient demographic and exposure data, the utility of this assay and its ready application in our laboratory workflow and result turnaround margins is already evident.

**INTRODUCTION**

*Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) is a common cause of gastrointestinal illness in the UK and worldwide. Approximately 3500 cases are reported annually in England and Wales to Public Health England (formerly the Health Protection Agency) (HPA, 2011), but this is an underestimate of the prevalence of disease (Ellam et al., 2008; Tam et al., 2012). Current methods employed by publicly funded primary diagnostic laboratories in the UK identify the presence/absence of the protozoan (HPA, 2012). Although this generally fulfils their diagnostic purpose, allowing appropriate clinical intervention, it offers less in terms of pursuit of cases’ relatedness and investigation of outbreaks, and identification of possible sources or transmission routes.

Molecular approaches to further discriminate *G. duodenalis* isolates are reliant on the efficient extraction of DNA, the target template, from cysts present in stools. Various successful approaches have been reported including use of a stool extraction kit (QIAamp; Qiagen) either with or without additional prior cyst disruption by freeze–thawing (Becher et al., 2004; Guy et al., 2004; Babaei et al., 2011), and bead beating followed by silica-based immobilization, washing and elution (Caccio et al., 2008). All of these approaches apply the basic principle of first disrupting the Giardia cysts by chemical lysis, heat, mechanical means or a combination of these, followed by extraction of DNA from the trophozoites within; the stool kit (Qiagen) appears to predominate in the literature.

Published typing studies have revealed that the *G. duodenalis* assemblages (genetic groups) most commonly found in human cases are A and B, with other assemblages found only very rarely (Feng & Xiao, 2011). Typing using conventional PCR and sequencing of the small subunit...
rRNA and triose phosphate isomerase (tpi) genes revealed that almost three-quarters of cases in south-west London, UK, were infected with assemblage B (Breathnach et al., 2010). A similar proportion was reported in Belgium using conventional PCR and sequencing of three genes: β-giardin, tpi and glutamate dehydrogenase (gdh) (Geurden et al., 2009). Others have used probe-based real-time PCR assays based on the β-giardin gene revealing a similar assemblage distribution in Canada (Guy et al., 2004). Melting-curve analysis of the tpi, gdh and ORF C4 loci revealed a high proportion of assemblages A and B (i.e. mixed) assemblages in stools from Italian and African cases (Almeida et al., 2010). A study in France demonstrated a predominance of assemblage A using conventional PCR and sequencing of the tpi and β-giardin genes, and offered the cautionary note that, due to high intra-assemblage heterogeneity, these loci may not be suitable choices for sequence alignment-based identification approaches (Bonhomme et al., 2011). Subassemblage identification using multi-locus genotyping has seen utility among assemblage A isolates but not those of assemblage B due to their higher sequence divergence. However, there is currently an absence of harmonized multi-locus genotyping methodologies and intra-assemblage nomenclature for either human infectious G. duodenalis assemblage (Bonhomme et al., 2011).

The significance of typing Giardia isolates from stools has yet to be realized as there have been few epidemiological studies incorporating these data; neither patient demographic influence nor the roles of anthroponotic or zoonotic transmission in the distribution of assemblages A and B have been fully elucidated (Feng & Xiao, 2011). Typing has been applied during the investigation of some giardiasis outbreaks. Cases linked to a UK day-care nursery (assemblage B), a foodborne outbreak in the USA (assemblage B) and waterborne outbreaks (two assemblage B and one assemblage A) in the USA and Canada, respectively, revealed a single responsible assemblage in each outbreak (Feng & Xiao, 2011). In Norway, typing data were used during investigation of a drinking water outbreak to exclude one suspected source of contamination (a septic tank) on the basis of detection of assemblage A, which was different from that isolated from the cases (assemblage B) (Robertson et al., 2006).

We have developed a Giardia typing protocol to estimate the distribution of Giardia assemblages A and B in giardiasis cases in Wales, UK, and to investigate the utility of including typing data in epidemiological investigations by analyses of patient demographics and exposure history. This involved evaluation of a suitable DNA extraction method and design, evaluation and validation of a real-time PCR assay, the whole process being capable of generating results within a single working day. The tpi locus was chosen as the target for our assay as it has previously shown promise in two studies that used conventional PCR (Geurden et al., 2009; Breathnach et al., 2010) and in a melting-curve analysis-based real-time PCR assay (Almeida et al., 2010). The relatively high nucleotide substitution rate observed at this locus offers variation appropriate for differentiation of assemblages A and B using real-time PCR, with the potential for differentiation within assemblages (i.e. subtyping) (Feng & Xiao, 2011). This locus has been used, alongside other loci, in the phylogenetic analysis of large datasets to confirm the genetic separation uniqueness of assemblages A and B (Feng & Xiao, 2011). In addition, the inter-assemblage sequence polymorphisms at this locus are suitable sites for the development of real-time primers and probes.

**METHODS**

**DNA extraction.** To contribute to estimates of DNA extraction efficacy, *G. duodenalis* cyst densities were first estimated in each of 15 *G. duodenalis* microscopy-positive stool samples by direct immunofluorescent microscopy (IFM) (Giardia-Cell; Cellabs) and recorded on an ordinal scale of 1+ (two to five cysts per field of view at ×400 magnification), 2+ (6–10 cysts per field), 3+ (11–50 cysts per field) and 4+ (>50 cysts seen per field). The IFM scores were compared with the threshold cycle (C_t) values from the real-time PCR using the Pearson correlation test in MS Excel.

To extract DNA directly from the 15 *G. duodenalis* microscopy-positive stools, samples were processed using three methods as described below.

Method 1: Stool (0.2 ml) was processed using a QIAamp DNA stool kit (Qiagen) according to the manufacturer’s instructions with the exception that lysis was carried out in 1 ml, rather than 1.4 ml, ASL buffer at 95 °C for 10 min. This adjustment was made because the available tubes suitable for high- and low-temperature incubations had a 1.5 ml vol., which was insufficient for 1.4 ml ASL buffer and 0.2 ml stool.

Method 2: As in method 1 but preceded by subjecting samples (in ASL buffer) to five freeze–thaw cycles using liquid nitrogen and a 100 °C heating block.

Method 3: (also known as the ‘Boom method’; Boom et al., 1999; McLauchlin et al., 1999), 0.2 ml stool was mixed with 900 μl L6 buffer containing guanidine thiocyanate (Severn Biotech), 20 μl isoamyl alcohol (Sigma) and 0.3 g 0.5 mm zirconia beads. Cysts were disrupted in a FastPrep-24 instrument (MP Biomedicals) for 1 min at 6.5 m s^{-1}. The DNA was extracted using a silica matrix, washed with ethanol and acetone, and finally eluted into 120 μl nuclease-free water.

Each DNA extract was amplified in triplicate using the real-time PCR typing assay developed as described below. To assess DNA yield, C_t values, which indicate the relative amount of DNA template prior to PCR by measuring the amplification cycle at which the fluorochrome achieves a predetermined amount or threshold, were compared, pairwise, between methods using a paired t-test (MS Excel) (Table 1).

**Co-extraction of substances inhibitory to PCR.** To compare the relative presence of substances inhibitory to PCR, potentially carried over from stool samples into the DNA extract, a commercial non-competitive internal control (IC) DNA target (Primer Design) was added to each PCR. Briefly, 1 μl IC primer-probe [labelled with 6-carboxyfluorescein (FAM)] mix and 5 μl 1:20-diluted IC DNA was amplified using 12.5 μl TaqMan Environmental Master Mix 2.0 (Applied Biosystems) in the presence of 3 μl DNA from each of the 15 samples extracted by each of the three methods described above. In addition, the IC was also amplified in duplicate tubes without Giardia...
Table 1. Attributes of the three *Giardia* spp. DNA extraction methods

Method 1, QIAamp stool kit + 95 °C for 10 min; method 2, five freeze–thaw cycles + QIAamp stool kit + 95 °C for 10 min; method 3, 'Boom method' (bead beating followed by liquid-phase silica extraction).

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of samples positive</td>
<td>15/15 (100%)</td>
<td>15/15 (100%)</td>
<td>15/15 (100%)</td>
<td></td>
</tr>
<tr>
<td><em>Giardia</em> DNA yield expressed as mean C&lt;sub&gt;i&lt;/sub&gt; value (range; n=15)</td>
<td>34.96 (29.93–39.99)</td>
<td>33.20 (28.83–38.91)</td>
<td>31.46 (27.43–37.84)</td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;i&lt;/sub&gt; values for assemblage A (n=2)</td>
<td>30.73, 39.15</td>
<td>32.10, 33.54</td>
<td>28.67, 31.10</td>
<td>All pairwise comparisons P&lt;0.01</td>
</tr>
<tr>
<td>Mean C&lt;sub&gt;i&lt;/sub&gt; value (range) for assemblage B (n=13)</td>
<td>34.76 (29.93–39.99)</td>
<td>33.25 (28.83–38.91)</td>
<td>31.70 (27.43–37.84)</td>
<td>M1:M2 P=0.51; M1:M3 P&lt;0.01; M2:M3 P&lt;0.01</td>
</tr>
<tr>
<td>IC mean C&lt;sub&gt;i&lt;/sub&gt; values (range) measuring extent of inhibition (n=15)</td>
<td>36.61 (34.3–43.53)</td>
<td>36.21 (34.7–39.43)</td>
<td>40.13 (35.60–50.11)</td>
<td></td>
</tr>
<tr>
<td>Relative cost of laboratory consumables</td>
<td>Medium</td>
<td>High</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Biocontainment</td>
<td>Sample captured on solid-state membrane in closed tube</td>
<td>Sample captured on solid-state membrane in closed tube</td>
<td>Multiple wash stages in liquid phase; open tube drying stage</td>
<td></td>
</tr>
<tr>
<td>Hands-on time</td>
<td>2.5 h</td>
<td>3 h</td>
<td>4 h</td>
<td></td>
</tr>
<tr>
<td>Ease of use</td>
<td>Kit-based method; easy to follow manufacturer’s instructions; laboratory staff familiar with method</td>
<td>Kit-based method, easy to follow manufacturer’s instructions; laboratory staff familiar with method</td>
<td>Multiple stages in method; in-house protocol with no company produced protocol</td>
<td></td>
</tr>
<tr>
<td>Numbers of samples in batch processing</td>
<td>Up to 20</td>
<td>Up to 20</td>
<td>Up to 28</td>
<td>Limited by heating block and FastPrep-24 capacities</td>
</tr>
<tr>
<td>Specialist equipment</td>
<td>None</td>
<td>None</td>
<td>Bead-beater or other cell disruptor</td>
<td></td>
</tr>
</tbody>
</table>

DNA. The mean C<sub>i</sub> values, which in the absence of inhibition would be the same for each extraction method, were compared, pairwise, using a paired t-test (MS Excel) (Table 1).

**Weighted multi-attribute comparison.** The following attributes of the three extraction methods were investigated: proportion positive by PCR, DNA yield measured by C<sub>i</sub> value, extent of inhibition, relative cost of laboratory consumables, biocontainment, hands-on time, ease of use, number of samples potentially processed per batch and any requirement for specialist equipment. Each method was ranked either by utility or subjectively on an ordinal scale from 1 (least desirable) to 3 (most desirable) for that attribute. Where methods shared the same utility, rank ‘2’ was awarded to each and either ‘3’ or ‘1’ awarded if another method remained, depending on utility.

The nine attributes were weighted according to their relative importance or desirability within our laboratory, with priority values totalling 1, as described by Tuli et al. (2010), assigned as follows: postivity was considered most desirable (priority value 0.2), followed by biocontainment, inhibitor carry over and DNA yield (sensitivity) (all 0.15); then time and the need for specialist equipment, which were considered to be equally desirable (0.1); relative cost (0.09), ease of use (0.04) and numbers of samples that could be processed in a batch (0.02) were less important for our investigations (the expected number of samples from Wales each week is less than five). The total weighted score for each DNA extraction method was calculated by summing the product of the utility rank score, multiplied by the desirability score for each attribute (see Table 3).

**Design and development of a real-time PCR of the tpi gene.**

**Control material.** The PCR assay was developed initially using DNA from standard *G. duodenalis* isolates, provided generously as follows: GS (assemblage B), WB (assemblage A) and P15 (assemblage E) by Karin Troell (National Veterinary Institute, Sweden); G113a (assemblage A), DAT, E+, DAC (all assemblage B), G114a (assemblage D) and assemblages C and F by Lucy Robertson (Norwegian School of Veterinary Science, Norway); and Portland-1 (assemblage A) by Claire Ling (Public Health England, UK).

To confirm the identity of assemblages identified from clinical samples by the newly developed assay, we sequenced 15 isolates using a 530 bp region of the *Giardia tpi* gene, a genus-specific assay that spans both the *G. duodenalis* assemblage A and B typing target regions described here (Sulaiman et al., 2003). Sequences were compared with those in GenBank to confirm their identity using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

**Primer and probe design.** PCR primers described previously by Almeida et al. (2010) were modified to accommodate the design and siting of a minor groove binding (MGB) TaqMan probe between each assemblage-specific pair (Table 2). Primers and new probes were designed using the BioEdit software program, version 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to create CLUSTAL W (BioEdit version 7.0.9.0, http://www.xbio.ncsu.edu/bioedit/bioedit.html) alignments of representative *Giardia* sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/). The Primer Express software program (Applied Biosystems) was used to calculate melting temperatures.
and check for undesirable inter- and intra-molecular binding. Primer and probe sequences were then checked for cross-reactions with non-target sequences on the GenBank database using BLAST. Assemble A primers amplified a 76 bp product and assemblage B primers an 82 bp product.

Amplification was carried out on a Rotorgene 6000 instrument (Corbett Research) using the following thermal cycling conditions: 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 s and 60 °C for 60 s.

Each 25 μl reaction contained 12.5 μl TaqMan Environmental Master Mix 2.0. Forward (GDAF and GDBF) and reverse (GDAR and GDBR) primers (Integrated DNA Technologies) were included at a concentration of 300 and 900 nM, respectively. Each MGB TaqMan probe (Applied Biosystems) was at a final concentration of 100 nM and labelled as follows: assemblage A specific (GDAT) VIC labelled and assemblage B specific (GDBT) FAM labelled. To each tube, 3 μl DNA was added. Each PCR run included Giardia DNA and a no-template control.

Data were collected from the green (FAM), yellow (VIC) and orange (ROX normalization dye) channels during each 60 °C annealing/extension phase. Post-run analysis was performed using Rotorgene 6000 software version 1.7 (Corbett Research). C_t values for each reaction were determined by the cycle at which the fluorescence plot crossed a standardized threshold of 0.05 normalized fluorescence units for both channels.

Analytical sensitivity and specificity. To estimate the potential analytical sensitivity of the assemblage A PCR assay, a 10-fold dilution series of assemblage A DNA (Portland-1) with between 3 ng and 3 pg DNA per PCR was prepared in nuclease-free water and amplified. Similarly, a 10-fold dilution series of assemblage B (GS isolate) in which the quantities of DNA were estimated to be 10 ng, 1000 pg, 100 pg and 10 pg per PCR was prepared and amplified.

Analytical specificity was assessed by testing a range of G. duodenalis assemblage isolates provided as described above and DNA from other organisms identified and extracted in our laboratory unless stated otherwise: Cryptosporidium parvum, Cryptosporidium hominis, Cryptosporidium meleagriris, Cryptosporidium ubiquitum, Cryptosporidium cuniculus, Cyclospora cayetanensis, Eimeria tenella, Entamoeba histolytica, Entamoeba dispar, Toxoplasma gondii, Saccharomyces cerevisiae, Homo sapiens (NEQAS 0703; National External Quality Assessment Service, Watford, UK), Ascaris sp. and two microsporidia (Encephalitozoon intestinalis and Enterocytozoon bieneusi).

Table 2. Real-time PCR primers and probes identified for the G. duodenalis tpi gene

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer/probe</th>
<th>Position (nt)*</th>
<th>Sequence (5’→3’)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. duodenalis assemblage A</td>
<td>GDAF‡</td>
<td>647–662</td>
<td>CATTGCCCTTTCCGCC</td>
</tr>
<tr>
<td></td>
<td>GDAR‡</td>
<td>702–722</td>
<td>CTGCCGCTGATCGCTCAACTG</td>
</tr>
<tr>
<td></td>
<td>GDAT</td>
<td>679–693</td>
<td>VIC-CCATTGCGGCAAACA-MGB-NFQ</td>
</tr>
<tr>
<td></td>
<td>GDAT</td>
<td>679–693</td>
<td>VIC-CCATTGCGGCAAACA-MGB-NFQ</td>
</tr>
<tr>
<td>G. duodenalis assemblage B</td>
<td>GDBF§</td>
<td>939–958</td>
<td>GATGAACGCAAGGCCAATAA</td>
</tr>
<tr>
<td></td>
<td>GDBR‡</td>
<td>996–1020</td>
<td>TCTTTGATTCTCAATCTCCCTT</td>
</tr>
<tr>
<td></td>
<td>GDBT</td>
<td>959–976</td>
<td>FAM-AATATTGCTGCAGCTCGAG-MGB-NFQ</td>
</tr>
</tbody>
</table>

*Nucleotide position relative to GenBank accession nos: L02120 (assemblage A) and L02116 (assemblage B).
‡MGB, minor groove binder; NFQ, non-fluorescent quencher.
§Modified from Almeida et al. (2010).
§As described by Almeida et al. (2010).

Table 3. Ranked and weighted attributes of the three Giardia spp. DNA extraction methods for a real-time PCR typing assay

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Desirability weighting</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR + samples (proportion)</td>
<td>0.2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Giardia DNA yield</td>
<td>0.15</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Inhibitor carryover</td>
<td>0.15</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Biocontainment</td>
<td>0.15</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Time</td>
<td>0.1</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Specialist equipment</td>
<td>0.1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Relative cost (per sample)</td>
<td>0.09</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Ease of use</td>
<td>0.04</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Samples in batch (n)</td>
<td>0.02</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Total weighted score*</td>
<td></td>
<td>1.95</td>
<td>2.06</td>
<td>1.72</td>
</tr>
</tbody>
</table>

*Sum of utility ranks x desirability weighting.
Detection of mixed infections. To establish whether a mixed infection of both assemblage A and assemblage B DNA could be detected in a single sample in disproportionate amounts, a two-way titration series, in 10 increments of 10% by volume, of one field isolate each of assemblage A and assemblage B DNA was created and amplified. The $C_v$ value of each assemblage in each mixture was recorded and compared using Pearson’s correlation test in MS Excel.

Validation of the real-time PCR assay. To validate the assay for clinical practice, 78 Giardia-positive stools submitted to the Cryptosporidium Reference Unit by primary diagnostic laboratories in Wales, in June to December 2012, were typed using the new probe-based tpi gene assemblage-specific real-time PCR assay. Stools were processed by the method that performed best in the multi-attribute weighted comparison (method 2, see results below) in batches of 18 samples, extracted with positive- and negative-control material and amplified alongside assemblage A and B controls, plus a non-template control. Validation of the assay’s performance was assessed for consistency of results of: (i) characterization of an internal quality control Giardia-positive stool sample, extracted and typed with every batch, (ii) negative extraction controls and (iii) PCR amplification of assemblage A and B DNA controls.

The positivity was compared with the routine diagnostic method (microscopy). The assemblage results were compared by the patients’ sex using a $\chi^2$ test and by age using a Mann–Whitney $U$ test (Epi-info version 6, Centers for Disease Control and Prevention, USA). A Mann–Whitney $U$ test was also used to compare $C_v$ values by case sex.

RESULTS

DNA extraction method comparison

PCR results revealed that, of the 15 stool samples used for comparing G. duodenalis DNA extraction methods, two were assemblage A and 13 were assemblage B.

Comparative analysis Each of the three extraction methods demonstrated 100% PCR positivity for the 15 samples tested, with mean $C_v$ values of 34.96, 33.20 and 31.46 for methods 1 to 3, respectively (Table 1). However, in one replicate from one sample (no. 10) and all three replicates from another (no. 13) extracted using method 3, both assemblages appeared to be present whereas only one assemblage was detected in all aliquots extracted from the same samples by the other two methods; high $C_v$ values for the four aliquots were obtained: 36.12 in the assemblage A-specific PCR in a sample considered to be assemblage B by the other two methods; and 39.13, 37.82 and 39.99 in the assemblage B-specific PCR in a sample considered to be assemblage A. Sequencing of the 530 bp fragment of the tpi gene from DNA extracted by Methods 2 and 3 revealed that, in sample no. 10, only the expected assemblage B sequence was present. However, in sample no. 13, method 3-extracted DNA contained sequences consistent with assemblages A and B, whereas in that extracted with method 2, only the expected assemblage A sequence was present.

The method 3 results were considered to be false positives in these respective samples, and only $C_v$ data from the ‘expected’ assemblage, i.e. that which was present in the DNA extracted by the other two methods, are used in the results shown in Table 1 and further analyses here. DNA extracted using method 3 had the lowest mean $C_v$ value, indicating the greatest yield of G. duodenalis DNA ($P<0.01$) (Table 1).

Due to the small number of assemblage A samples ($n=2$), detailed comparisons of the effect of assemblage on $C_v$ values within and between tests is not indicated, although the $C_v$ values were within the range of those expected for assemblage B (Table 1).

The IFM scores from the 15 stool samples ranged from 1 to 4 + (1+, n=2; 2+, n=4; 3+, n=6; 4+, n=3) and had a negative correlation with $C_v$ values. The relationship was best for method 1 ($r=-0.76$), followed by method 2 ($r=-0.62$) and method 3 ($r=-0.55$).

Co-extraction of substances inhibitory to PCR The mean $C_v$ values for the amplified IC DNA in the presence of Giardia DNA extracted differed significantly. The mean $C_v$ value for the amplified IC DNA in the stool extracts prepared by method 3 (40.13) was significantly higher than methods 1 and 2 (36.61 and 36.21, respectively) ($P<0.01$), indicating greater inhibition of the IC PCR in samples extracted by method 3 (Table 1). The duplicate tubes in which no Giardia was inoculated had a lower $C_v$ still (33.63), which indicated that either all methods allowed substances inhibitory to PCR to carry over into the extracted DNA, or that in the absence of Giardia DNA, the IC DNA amplified more efficiently as it was not subjected to competition for amplification reagents.

Weighted multi-attribute comparison The utility rank score for each attribute of each extraction method is shown in Table 3. Multi-attribute comparison of the three methods resulted in method 2 having the highest, most desirable, score. Method 2 offered better DNA yields with lower inhibitor carry over, which, on samples containing low numbers of organism in particular, contribute to positivity and therefore whether the test would yield a typing result or not.

Design and development of a real-time PCR of the tpi gene

Confirmation of assemblage identity The assemblages identified by the real-time PCR were confirmed in all seven samples by DNA sequencing of the genus-specific PCR product: four assemblage A and three assemblage B.

Analytical sensitivity and specificity Assemblage A DNA was detectable in the lowest dilution preparation tested (3 pg per PCR). This equated to 2.32 x 10⁴ cyst genomes and therefore 14 cysts per PCR. Similarly, assemblage B DNA was detectable in the 1:1000-dilution preparation (10 pg per PCR). This equated to 16 cysts per PCR. The potential analytical sensitivity of the assemblage A PCR was estimated to be ~2400 cysts (g stool)⁻¹ and that of the
assemblage B PCR to be ∼2600 [or 10^3 cysts (g stool)^{-1} for both assays].

No cross-reactions with other genera were detected using the G. duodenalis assemblage primers and probes described here. Only the corresponding assemblage A or B DNA templates were amplified by the assemblage-specific assays. G. duodenalis assemblages C, D, E and F were not amplified using the real-time assay.

Detection of mixed infections DNA from assemblage A was detectable in the presence of up to nine times (by volume) as much assemblage B DNA, and vice versa (data not shown). The C_t values for each assemblage decreased as the proportion of that assemblage’s DNA increased in comparison with the decreasing proportion of the other assemblage, with a correlation value \( r = -0.74 \).

Evaluation of real-time PCR assay DNA was extracted from 78 G. duodenalis microscopy-positive stools using method 2, and typing was carried out using the new probe-based tpi gene assemblage-specific real-time PCR assay.

All of the 78 stools tested were PCR positive: 20 (26 %) were assemblage A, two (3 %) were assemblage A and B and 56 (72 %) were assemblage B. Most of the cases, 47/78 (60 %), were male; the mean age of all cases, where stated, was 31 years (n=77, median 34 years, range 1–84 years). A notable excess of cases with assemblage A were male (16/20, 80 %) compared with 30/56 (54 %) male assemblage B cases (\( \chi^2 = 4.25, P = 0.04 \)). The mean age of cases with each assemblage did not differ: assemblage A, mean 28 years, median 29 years, range 1–84 year; assemblage B, mean 32 years, median 35 years, range 1–65 years, \( P = 0.53 \); both assemblages, mean 31 years, median 34 years, range 1–84 years. Mean C_t values for all cases did not vary according to the sex of the case (females=29.1, males=29.5, \( P = 0.59 \)).

DISCUSSION

We have described the development of a G. duodenalis typing protocol that allows the reliable identification of the two main human-infective assemblages from stool samples within a single working day. Neither the DNA extraction nor the PCR assay requires specialized equipment that would not be commonly encountered in a molecular biology laboratory. In fact, the widely applicable reagents and the steady signal from the ROX dye, which is used for normalization in some real-time systems (but not required by the Rotorgene), suggests that the assay can be readily transferred to other real-time platforms.

Our choice of the tpi locus for the development of this real-time assemblage-specific typing assay is supported by the data presented here; the sensitivity and specificity are suitable for typing routinely identified Giardia-positive samples and can be applied to any real-time platform. We were able to identify the assemblage present in 100 % of the 78 microscopy-positive stools tested, which compares well with 70 % of 240 (Breathnach et al., 2010) and 97 % of 74 isolates (Geurden et al., 2009) from published conventional assays using this locus. Both the assay’s typability, and its potential analytical sensitivity, which showed that DNA equivalent to 10^3 cysts g^{-1} was detectable and typeable, confirm that this assay is suitable for its intended purpose. While the analysis of a second locus to confirm the typing result from the first may be considered desirable, it is impractical in the context of a routine typing service as it would incur further time and expense. The validation of the real-time PCR and inclusion of control material in each assay batch and close attention to quality control provide the necessary quality assurance.

Comparison of C_t values from each extraction method, indicating the relative amount of G. duodenalis DNA extracted, showed that, although method 3 resulted in significantly higher DNA yields, it was more susceptible to potential PCR inhibitors than the other two methods, which utilized a solid-state silica-based column. Further elimination of the negative effects of carried over inhibitors was accomplished using an environmental PCR master mix that has been developed specifically for target detection in the presence of high levels of inhibitors (Applied Biosystems). The finding that two of the 15 samples extracted using method 3 were positive for both assemblage A and B is surprising when compared with results from the other two extraction methods. The C_t values of the less abundant assemblage in these two samples indicated that, had these been genuine results, they would have been present in sufficient quantity to have been detected in all three extraction assays. We can say this with some certainty as the potentially contaminating assemblage was detected in these two samples at C_t values well before the end of the programme, even allowing for the apparent C_t lag of two to three cycles indicated in methods 1 and 2 compared with method 3. Furthermore, sequencing of the 530 bp tpi gene revealed sequences consistent with the presence of both assemblages in sample no. 13 extracted using method 3, but not method 2. The absence of mixed sequences in the other affected sample, no. 10, in which only one PCR replicate out of three contained both assemblages, is likely to be due to a low level of contamination that was not detected in the single DNA sample we sequenced. Therefore, it is possible that cross-contamination of the DNA occurred during extraction, a potential problem with method 3, which includes a drying stage in open tubes, compared with methods 1 and 2 where material is held on an enclosed membrane in nearly half of the stages. Method 2, using a QIAamp DNA Stool kit preceded by freeze–thaw treatment, offered efficient DNA extraction alongside better biocontainment and lower potential for inhibitor carryover. Although we did not undertake systematic evaluation of both disruption methods (bead beating and freeze–thaw) in combination with each method for DNA extraction, previous data show this was unlikely to be fruitful.

In one study, when bead beating was used before a Qiagen Stool kit extraction, 5 % of samples had to be re-extracted using a different method (Lebbad et al. 2011).
The analysis of 78 samples typed in the validation of our assay revealed comparable data to that generated from human cases both elsewhere in the UK and internationally, the proportion of assemblage A and B cases being very similar to that reported in London, UK, by Breathnach et al. (2010), with roughly one-quarter assemblage A and three-quarters assemblage B. The overall finding globally is that assemblage B appears to predominate (Feng & Xiao, 2011). The sex distribution of the cases described here differed from those described by Breathnach et al. (2010) who reported an approximately equal distribution both in all cases and by assemblage, whereas the smaller number of Welsh cases tested so far showed a disproportionate number of male cases overall and having assemblage A.

The development of timely typing protocols such as ours is an important step in assisting public health investigations of case relatedness, and wider epidemiological studies that may reveal assemblage-specific associations. Our understanding of G. duodenalis transmission may improve through increasing the resolution of typing techniques allowing the potential development of control measures to eventually reduce the public health burden of this important parasite.

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