Summary:

A cDNA clone encoding GLORF-C4 was isolated from the WB strain, an assemblage A Giardia intestinalis. Interestingly, GLORF-C4 has been previously reported as an assemblage B-specific gene. Using two primers based on GLORF-C4 of the GS strain, a prototype assemblage B, GLORF-C4 gene was amplified from all the groups of G. intestinalis, and applied to detect the presence of cysts of G. intestinalis from faecal samples of cyst-passers. RFLP analysis of this PCR product successfully classified G. intestinalis into two distinct groups, assemblages A and B.

KEY WORDS: Giardia intestinalis, GLORF-C4, PCR-RFLP.

PCR-RFLP ANALYSIS OF GIARDIA INTESTINALIS USING A GIARDIA-SPECIFIC GENE, GLORF-C4

YONG T.*, HAN K.*, YANG H.* & PARK S.*

Giardia intestinalis is a significant cause of diarrhoeal outbreaks worldwide. On the basis of the shared morphological characteristics, it includes a wide range of organisms, which are ubiquitous in various mammalian hosts. Because of its zoonotic potential, classification of this organism has been an important issue, which may provide clues to monitor possible sources for the outbreaks as well as to identify the routes of infections. For categorising G. intestinalis, several modes of classification have been employed. Two major lineages have been defined for analysis of human-derived G. intestinalis, assemblages A and B (Mayrhofer et al., 1995), which correspond either to the ‘Polish’ and the ‘Belgian’ genotypes (Homan et al., 1992), or to the groups I plus II and the group III plus IV (Andrews et al., 1989; Monis et al., 1996), respectively. Alternatively, diverse G. intestinalis has been classified into three groups, 1, 2, and 3 (Nash, 1985; Nash et al., 1985, 1990). It has been suggested that groups 1 plus 2 and group 3 correspond to assemblage A and assemblage B, respectively (Lu et al., 1998). Analysis of animal-derived G. intestinalis identified three additional assemblages, assemblage C and D recovered from dogs (Monis et al., 1998), and assemblage E (a “Novel livestock” lineage) defined by Ey et al. (1997). Recent phylogenetic investigation using four housekeeping genes and allozyme analysis showed that G. intestinalis includes at least seven different lineages, assemblage A to G (Monis et al., 1999).

In this report, we found a cDNA clone of G. intestinalis showing a slight increase in transcription during encystation process. Despite of the minor increase in transcription of this cDNA, it attracted our attention in that this cDNA clone isolated from WB isolate, an assemblage A strain, turned out to be GLORF-C4, previously reported as the assemblage B-specific gene from the GS strain (Nash & Mowatt, 1992). Moreover, no homologous gene for this cDNA has yet been identified in other organisms.

Finding of GLORF-C4 in the WB isolate led us to investigate whether this gene is prevalent in different groups of G. intestinalis. We also asked if this unique gene could be applied to detect and subgroup the G. intestinalis obtained from the faecal samples.
MATERIALS AND METHODS

CLONING OF GLORF-C4 FROM THE WB STRAIN

For the cDNA of 250 bps, showing a slight increase in transcription during in vitro encystation, the nucleotide sequence was compared with EMBL and GenBank databases using the Blast program. To find a full gene for this cDNA, we also carried cDNA library screening. For that purpose, G. lamblia cDNA library constructed by Prof. Chung D. (Kyoungbook National University, Korea) was employed. Using this A/ZAPII-based cDNA library (Stratagene), clones hybridizing with the 32P-labeled cDNA were purified until homogeneity and rescued as plasmids as directed by manufacturer. Identities of the isolated cDNAs were verified using an automatic DNA sequencer (Pharmacia).

CELL CULTIVATION AND DNA PREPARATION

G. intestinalis WB (ATCC#30957) and GS (ATCC#50581) strains were axenically cultivated using TYI-S-33 media (Keister, 1983). An axenic culture of YS-27 strain (Chang et al., 1995) was used as a source for genomic DNA of Entamoeba bistolytica. Escherichia coli DH5a and Vibrio vulnificus (ATCC#29307) were propagated in Luria broth with different saline concentrations (1, 2 %, respectively). Acanthamoeba culbertsoni (ATCC#30171) was cultured in CGV medium (Willaert, 1971) and used as a source for genomic DNA.

DNAs of the above strains were extracted by phenol extraction as described (Park et al., 1999). Genomic DNA of Toxoplasma gondii RH strain was a gift of Prof. Ahn M. (University of Hanyang, Korea).

PCR AMPLIFICATION USING PROBES FOR G. INTESTINALIS

Genomic DNAs from G. intestinalis WB and GS strains were employed as templates for PCR with diverse primers. PCR amplification was performed using a GeneAmp PCR System 9600 (Perkin-Elmer). The PCR solutions contained 10 mM KCl, 8 mM (NH4)2SO4, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.01 % (w/v) gelatin, 100 µM of each deoxyribonucleotide, 20 pmol of each primer, 1 Unit of Taq polymerase (Takara) and 50 to 200 ng of template DNA. Primers used in this experiment are listed in Table I.

For SSU-rDNA specific primers, RH4-RH11 (Hopkins et al., 1997) and JW1-JW2 (Weiss et al., 1992), the reactions were proceeded by 35 cycles comprised with the following three steps: denaturation at 94°C for 30 sec, hybridization at 59°C for 1 min, and polymerization at 72°C for 30 sec. In the reaction mixtures, glycerol and dimethylsulfoxide were included at final concentrations of 10 % and 5 %, respectively. In case of tim-specific primers (7493-5945 and 4130-4131: Baruch et al., 1996), a total of 35 PCR cycles were carried out during which DNAs were denatured at 94°C for 30 sec and the primers were annealed at 55°C for 1 min and extended at 72°C for 1 min. For GGL and GGR primers for the giardin gene, PCR was performed by 25 cycles of amplification made of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. GLORF-C4 was amplified with C4-F and C4-R under the following PCR condition (35 cycles: 94°C, 30 sec, 58°C, 1 min, 72°C, 1 min).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Nucleotide sequence</th>
<th>Size (bps)</th>
<th>Reference</th>
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<td>GLORF-C4</td>
<td>C4-F</td>
<td>5'AGCTCATCTTCTGCTCCTCTA3'</td>
<td>443</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>C4-R</td>
<td>5'CAATCTTGTGCTCGATACGGA3'</td>
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<tr>
<td>giardin</td>
<td>GGL</td>
<td>5'AAGTGCGTCAAGGACGCTG3'</td>
<td>171</td>
<td>Mahbubani et al. (1992)</td>
</tr>
<tr>
<td></td>
<td>GGR</td>
<td>5'TTAGTGCTTGTGACACCAGGAA3'</td>
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<td>Baruch et al. (1996)</td>
</tr>
<tr>
<td></td>
<td>4131</td>
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<td>812</td>
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</tr>
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<td>4130</td>
<td>5'CAGCTGCCAAGCTTGCTCAGG3'</td>
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<td></td>
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<td>7493</td>
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<td></td>
<td>5945</td>
<td>5'TAGCTCCTGCGTCTGCTG3'</td>
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<tr>
<td>SSU-rDNA</td>
<td>RH11</td>
<td>5'CATCCGCTCAGTCTCCTGCC3'</td>
<td>292</td>
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<td>RH4</td>
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<td>165</td>
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<td>JW1</td>
<td>5'GGCGACACAGGAAATGTCTTG3'</td>
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<tr>
<td></td>
<td>JW2</td>
<td>5'TCACCACGGATACCGTGT3'</td>
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</tr>
</tbody>
</table>

Table I. – Oligonucleotide primers used in detection of Giardia intestinalis.
PCR detection of *G. intestinalis* from faecal samples

*Giardia*-positive samples identified by light microscopy were processed to purify genomic DNA by sucrose gradient centrifugation and subsequent phenol extraction, as previously described (Yong *et al.*, 2000). Using these genomic DNA as templates, presence of *G. intestinalis* was examined by PCR reaction (35 cycles: 94°C, 30 sec, 58°C, 1 min, 72°C, 1 min).

**RESULTS**

**Sequence of GLORF-C4 in WB strain**

Sequence data of GLORF-C4 gene of the WB strain were submitted to EMBL databases and obtained the accession number AJ291756. The nucleotide sequence of GLORF-C4 in WB was aligned with that in GS and found to have 73 % identity (Fig. 1). Both deduced amino acid sequences show 72 % identity (data not shown). This result is intriguing in that it demonstrates a significant variation in the same gene within the same species. Due to this variation, GLORF-C4 might have been suggested as an assemblage-specific gene, which seemed to be present only in the assemblage B, but not in the assemblage A (Nash & Mowatt, 1992).

**Determination of specificity of GLORF-C4**

For comparison, WB and GS strains were employed as prototypes for the assemblages A and B, respectively. Two primers, C4-F and C4-R were used to amplify a partial sequence of this gene as a 443 bp DNA fragment from the two strains of *G. intestinalis*, as well as from the other organisms.

As a result of PCR on the genomic DNAs isolated from WB and GS, the DNA fragments of 443 bps were amplified. On the contrary, PCR with the genomic DNAs of other protozoa (*Acanthamoeba culbertsoni, Entamoeba histolytica, and Toxoplasma gondii*) and the bacteria (*Escherichia coli* and *Vibrio vulnificus*) failed to produce any PCR product (Fig. 2). This result clearly demonstrated the unique presence of GLORF-C4 in *G. intestinalis*.

**Comparison of GLORF-C4 with other probes**

Based on this unique prevalence of GLORF-C4 within *G. intestinalis*, it was compared with other genes

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**Fig. 1.** - Aligned nucleotide sequences of the GLORF-C4 gene of *G. intestinalis* isolate WB (assemblage A genotype; EMBL accession number AJ291756) and its putative ortholog (GenBank accession number M90390) from isolate GS, an assemblage B genotype. The segments corresponding to PCR primers C4-F and C4-R are indicated by arrows.
(Mahbubani et al., 1992; Weiss et al., 1992; Baruch et al., 1996; Hopkins et al., 1997), which had been reported as a probe to detect *G. intestinalis* (Table I).

Upon PCR employing two pairs of the primers corresponding to SSU-rDNA region, JW1 plus JW2 (Weiss et al., 1992) and RH11 plus RH4 (Hopkins et al., 1997), the DNA fragments of the expected sizes, 163 and 292 bps, were obtained (Fig. 3A). When the primers complementary to the region of giardian gene, GGL and GGR (Mahbubani et al., 1992) were used, a DNA fragment of 171 bps was generated from both isolates. These three sets of primers mentioned above have been applied to detect *G. intestinalis* from clinical and environmental samples.

PCR with two pairs of *tim*-specific primers (4130 plus 4131 and 7493 plus 5945; Baruch et al., 1996) resulted in production of 683 and 812 bp DNA fragments, respectively (Fig. 3A, lane 8-11). When these two DNA fragments were digested with either *Xho* I (for 683 bp DNA) or *Hind* III (for 812 bp DNA), WB and GS showed different digestion patterns (fig. 3B), thus providing a tool to classify *G. intestinalis*.

We also tested whether the GLORF-C4 PCR fragment could be used to categorise different groups of *G. intestinalis* by PCR-RFLP. Digestions of the GLORF-C4 PCR fragments with various restriction endonucleases, such as *Nco* I, *Apa* I, *Hind* III, or *Tru*9 I, provided an straightforward way of distinguishing the assemblage B from the assemblage A strain (data not shown). Differential digestion patterns between WB and GS isolates with *Tru*9 I or *Nco* I are presented in Fig. 3B.

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**Fig. 3.** (A) PCR detection of *Giardia intestinalis* WB (assemblage A) and GS (assemblage B) strains using various primers, SSU-rDNA primers, *tim* primers, or GLORF-C4 primers. W represents WB whereas G indicates GS isolate. Using these isolates as the templates, PCR was performed using the following primers. Lane 1, DNA size marker, 100 bp ladder; lane 2, 3, JW1 and JW2 (for SSU-rDNA); lane 4, 5, RH11 and RH4 (for SSU-rDNA); lane 6, 7, GGL and GGR (for giardin); lane 8, 9, 4130 and 4131 (*tim*); lane 10,11, 5925 and 7493 (*tim*); lane 12, 13, C4-F and C4-R (*tim*) for GLORF-C4).

(B) Grouping of *Giardia intestinalis* using *tim*-specific primers and GLORF-C4 primers. W represents WB whereas G indicates GS isolate. Using these isolates as templates, reactions were carried out with the following primers and the resultant PCR products were digested with indicated restriction endonucleases, lane 1, DNA size marker, 100 bp ladder; lane 2, 3, 4130 and 4131 (*Xho* I); lane 4, 5, 5925 and 7493 (*Hind* III); lane 6, 7, C4-F and C4-R (*Tru*9 I); lane 8, 9, C4-F and C4-R (*Nco* I).

---

**APPLICATION OF C4-F AND C4-R TO FECAL SAMPLES**

C4-F and C4-R primers were employed to detect *G. intestinalis* in the faecal samples obtained from Korea, China, and Laos (Table II). PCR reactions using these genomic DNAs as templates produced the same 443 bp DNA fragment (data not shown). Eight of the fourteen samples have been previously grouped by a different method, sequencing the 292 bp region of SSU-rDNA (Yong et al., 2000).
PCR-RFLP analysis of GLORF-C4 PCR products from human faecal samples showed that three samples isolated from Korea belong to the assemblage A, as shown two products of 303, and 140 bps. For the faecal samples from China, both patterns of digestion were demonstrated, two as an assemblage B type (an intact 443 bp DNA fragment) and four as an assemblage A type. Five samples from Laos were turned out to be assemblage B isolates (Fig. 4 and Table II). PCR-RFLP results of GLOFR-C4 were summarised with the sequencing data of SSU-rDNA for comparison, demonstrating that both types of analyses resulted in the same groupings.

**DISCUSSION**

Classification of *G. intestinalis* has become more important question as this organism attracted worldwide attentions as sources for outbreaks. Elucidation of the contaminating sources and monitoring and surveys of the possible sources are the prerequisites for prevention of the outbreaks by this protozoan pathogen. In this note, we designed a set of primers specific for GLORF-C4, which was shown to be unique to *G. intestinalis*. Compared with two pairs of SSU-rDNA primers and a pair of primers for giardin, C4-F and C4-R primers provide a better way to classify *G. intestinalis*. In case of the former primers, sequencing of the PCR products is required to identify the groups of *G. intestinalis*. With *r/w*-specific primers, classification of *Giardia* isolates was feasible with PCR-RFLP. Actually, PCR-RFLP analysis of *tim* fragment has an advantage over that of GLORF-C4 in that the former method allows the sub-grouping of *G. intestinalis* assemblage A into two sub-assemblages A-1 and A-2 (Baruch *et al.*, 1996). On the other hand, we found that digestion patterns of GLORF-C4 of WB isolate (assemblage A-1) was exactly same as those of K1 isolate, which has been reported as an assemblage A-2 (*Park et al.*, 1999) (data not shown). Therefore, sub-grouping of assemblage A is not possible with PCR-RFLP analysis of GLORF-C4. Despite of this ability, PCR-RFLP analysis of *tim* fragments needs two PCRs to identify the groups of the isolates. On the other hand, PCR-RFLP analysis of GLORF-C4 could be suggested as an improved, convenient method in which a single PCR product treated with either *Nco* I or *Tru*9 I can provide a comple-

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Sampling site</th>
<th>Grouping by 16S rDNA</th>
<th>Grouping by GLORF-C4</th>
</tr>
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<td>KP</td>
<td>Seoul, Korea</td>
<td>Assemblage A</td>
<td>Assemblage A</td>
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<tr>
<td>KL</td>
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<td>Assemblage A</td>
<td>Assemblage A</td>
</tr>
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<td>Assemblage A</td>
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<td>Assemblage A</td>
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<td>CA9</td>
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<td>RE19</td>
<td>Savannakhet, Laos</td>
<td>nd</td>
<td>Assemblage B</td>
</tr>
</tbody>
</table>

* Data from Yong *et al.* (2000).

**Table II.** Summary of the faecal samples used in RFLP analysis.

![Fig. 4. - PCR-RFLP analysis of GLORF-C4 isolated from human faecal samples. The GLORF-C4 DNA fragments amplified from various isolates were digested with *Tru*9 I endonuclease. Lane 1, 100 bp ladder; lane 2, WB (assemblage A); lane 3, GS (assemblage B); lane 4-6, the Korean sample, KP, KL, KC2; lane 7-9, the Chinese samples, CA13, CA14, CA17; lane 10-11, the Laos samples, RD25, and RE19.](image)
acknowledgment, confirming result in aspect of the grouping of *G. intestinalis*.

Lastly, we also applied our primers to the faecal samples from three different countries and found them useful to detect and classify *Giardia* isolates. Due to the small number of the faecal samples analysed, our data is preliminary to make any conclusion on distribution of two assemblages in these countries. However, a suggestion can be proposed based on two facts: 1) a proposition made in SSU-rDNA analysis (Yong et al., 2000), which *G. intestinalis* of assemblage B is phylogenetically grouped closer to *Giardia* isolates obtained from animals, 2) grouping of Laos isolates only to the assemblage B. Comparing with China and Korea, there may be a higher probability of cross-transmissions between humans and animals in Laos. To confirm this, more investigation should be performed including a large number of human faecal samples as well as animal-originated samples.

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**REFERENCES**


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