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

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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.

Résumé : ANALYSE EN PCR-RFLP DU GÈNE SPÉCIFIQUE GLORF-C4 DE *GIARDIA INTESTINALIS*

Un clone d'ADN complémentaire codant pour le gène GLORF-C4 qui était considéré comme spécifique des souches de l'assemblage B a été isolé de la souche WB (de l'assemblage A) de Giardia intestinali. *Ce gène GLORF-C4 a été amplifié à partir de tous les groupes de* G. intestinalis *en utilisant des oligonucléotides spécifiques du gène GLORF-C4 de la souche GS, prototype des souches de l'assemblage B, et utilisé pour détecter la présence de cystes de* G. intestinalis *dans les matières fécales de sujet excrétant des cystes. L'analyse en RPLF de ces produits d'amplification a permis la classification des différents groupes de* G. intestinalis *en deux catégories distinctes : celle de l'assemblage A et celle de l'assemblage B.*

MOTS CLÉS : Giardia intestinalis, *GLORF-C4, PCR-RFLP.*

Giardia intesinalis is a significant cause of diarrheal 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.

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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 λZAPII-based cDNA library (Stratagene), clones hybridizing with the ^{32}P-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 histolytica. Eshcerichia 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 $(NH_4)_2SO_4$, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 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).

Gene	Primers	Nucleotide sequence	Size (bps)	Reference
GLORF-C4	C4-F	5'AGCTCATCTTCGTCCTCTA3'	443	This study
	C4-R	5'CAATCTTGTTTGCATACGA3'		
giardin	GGL	5'AAGTGCGTCAACGAGCAGCT3'	171	Mahbubani *et al.* (1992)
	GGR	5'TTAGTGCTTTGTGACCATCGA3'		
tim	4131	5'ATGCCTGCTCGTCGCCCCTTC3'	683	Baruch *et al.* (1996)
	4130	5'CACTGGCCAAGCTTCTCGCAG3'		
	7493	5'GCAGAATGTGTACCTAGAGGGG3'	812	Baruch *et al.* (1996)
	5945	5'TAGTCTCCGAGCTCCTTCTGG3'		
SSU-rDNA	RH11	5'CATCCGGTCGATCCTGCC3'	292	Hopkins *et al.* (1997)
	RH4	5'AGTCGAACCCTGATTCTCCGCCAGG3'		
	JW1		163	Weiss *et al.* (1992)
	JW2	5'GCGCACCAGGAATGTCTTGT3'		
		5'TCACCTACGGATACCTTGTT3'		

Table I. – Oligonucelotide 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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GS    ATGTTCAACCCGAGACACCCTGGAGGCGAATTCTTTGGAAGAAAGCACCACCGAAGGCAT
WB    ATGCACGAGCCAAGGCACGCCAACGGCGAGTTCTTTGGAAGGAGGCATCATGGAAGGCGT

                          C4-F
                      ------------>

GS    GCGCCAGATGGAAGATCTAGCTCATCTTCGTCCTCTAGCTCTGAGTGCGAGCTTGGCTAT
WB    CCACCGGATGAAAGCTCCAGCTCATCATCGTCCTCTAGCTGTGAGTGCGAAGCTGGCCAT

GS    GACGAAAGAAGATGCTGTAGGCAGGAGCCGGATGCTCATTGTTGCGATCGCGAGCATCGT
WB    GATGAAAGGGAGTGCTGTAGACAGGGCCCAGGCCCATACTGCTGCGACCGCGAGCGCCGC

GS    AAATTCTGGAAGCACCCGCCTGAATTCCCTCACTTTGGCAAGTGTAAGGGACGTCATGCG
WB    AAATTCTGGAAACACGGGCCCCCGTTCTCCCACTTTATTAAGGGCAGGGGACATCATGGA

GS    CTTCCTTTCCCAAGGATGGAGCACTGGCCTGGCCCCAAGCACTGTCCATTTCTATCTGAG
WB    CCCCACTGTTCAAAAGAAGAGCGCTGGACCGGTCCCAAACCCTGCCCATTTCTGTCATCC

GS    AAGGACATGAAGGACCTTCCTGTTACTGTTCACCATGGGTGCATGCCTATTGTTGCTCAC
WB    AAAGATATGAATGAGCTTCCTGTCACCGTTCATCACGGAAGCATGCCGATTGTCGCACGT

GS    GGAGTGGTCATGTTTCACATAGTATCCGAAACAAAGCCGTCCATAACCATCTCTAACACT
WB    GGAGTGGTCACGTTTCATATTGTATCAGAAATAAAGCCGTCCATAACAATCTCCAATACC

GS    GGCGCCATCAAGGTGGAGGCCAATGGAATCCAGCTTCTCAGTGCTTCTCTGAAGGAGAAG
WB    GGCGCCATCACTGTAGAGGCAGATGGAATCAAGCTTCTTAGCGCCTCCCTGAAGGAAAAT

                          C4-R
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GS    TACCCCGTCGATAAGGTCGTCGCATCGTATGCAAACAAGATTGCCACCATCCGTATTCCA
WB    TATCCTGCTGATAAGGTCGTTGCGTCGTATGCAAACAAGATTGTCGTTATCCGTATTCCT

GS    TGTGATGCGGCCTTCTCTGAGTCTCCTGTGGATATCCCTATCACGATCCAAAAGTGA
WB    TGCAACATAGTCACACCTGAGCCTTCTGTTGATATTCCTATTACGATTCAGAAGTAA
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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.

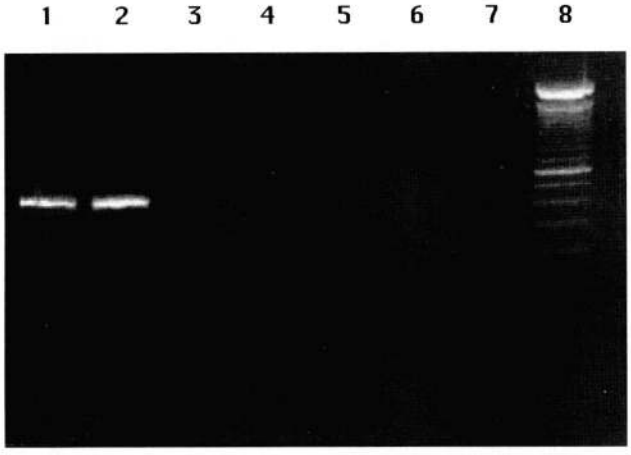

Fig. 2. – PCR detection of GLOFR-C4 gene in two groups of *Giardia intestinalis*, other protozoa and bacteria.
Using two primers, C4-F and C4-R based on GLORF-C4 sequence of GS, GLORF-C4 DNA of 443 bps were amplified from the following genomic DNAs; lane 1, WB (assemblage A *Giardia intestinalis*); lane 2, GS (assemblage B *Giardia intestinalis*); lane 3, *Acanthamoeba cubertsoni*; lane 4, *Entamoeba histolytica*; lane 5, *Toxoplasma gondii*; lane 6, *Escherichia coli* DH5a; lane 7, *Vibrio vulnifucus*; lane 8, DNA size marker, 100 bp ladder.

(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 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 *Hin*d 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, *Hin*d III, or *Tru9* 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 *Tru9* I or *Nco* I are presented in Fig. 3B.

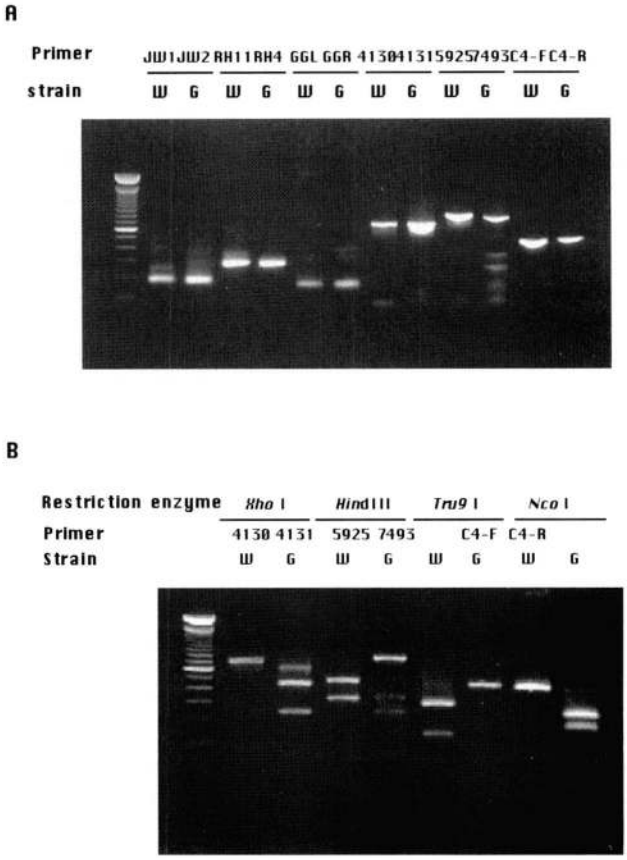

Fig. 3. – (A) PCR detection of *Giardia intestinalis* WB (assemblage A) and GS (assemblage B) strains using various primers, SSU-rDNA primers, giardin primers, *tim* primers, or GLOFR-C4 primers. W represents WB whereas G indicates GS isolate. Using these isolates as the templates, PCR was performed 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 (for *tim*); lane 10,11, 5925 and 7493 (for *tim*); lane 12, 13, C4-F and C4-R (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 (*Hin*d III); lane 6, 7, C4-F and C4-R (*Tru9* I); lane 8, 9, C4-F and C4-R (*Nco* I).

Application of C4-F and C4-R to faecal 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).

Sample number	Sampling site	Grouping by 16S rDNA[a]	Grouping by GLORF-C4
KP	Seoul, Korea	Assemblage A	Assemblage A
KL	Seoul, Korea	Assemblage A	Assemblage A
KC2	Seoul, Korea	Assemblage A	Assemblage A
CA1	Anhui, China	Assemblage A	Assemblage A
CA9	Anhui, China	Assemblage B	Assemblage B
CA12	Anhui, China	nd[b]	Assemblage A
CA13	Anhui, China	Assemblage A	Assemblage A
CA14	Anhui, China	Assemblage A	Assemblage A
CA17	Anhui, China	Assemblage B	Assemblage B
RD2	Savannakhet, Laos	nd	Assemblage B
RD25	Savannakhet, Laos	nd	Assemblage B
RD37	Savannakhet, Laos	nd	Assemblage B
RE4	Savannakhet, Laos	nd	Assemblage B
RE19	Savannakhet, Laos	nd	Assemblage B

[a] : Data from Yong *et al.* (2000).
[b] : nd, not done.

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

Tru9 I digestion 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.

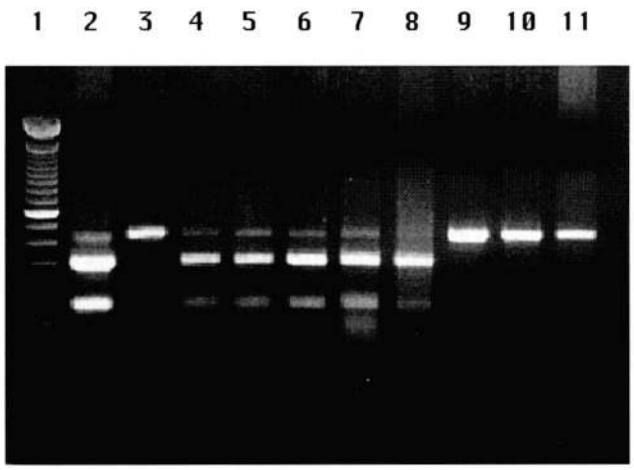

Fig. 4. – PCR-RFLP analysis of GLORF-C4 isolated from human faecal samples. The GLORF-C4 DNA fragments amplified from various isolates were digested with *Tru9* I endonuclease. Lane 1, 100 bp ladder; lane 2, WB (assemblage A); lane 3, GS (assemblage B); lane 4-6, the Korean sample, KP, KL, KC2; lane 7-9, the Chinese samples, CA13, CA14, CA17; lane 10-11, the Laos samples, RD25, and RE19.

DISCUSSION

Classification of *G. intesitnalis* 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*. They were compared side by side with other probes, known to detect and classify *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 *tim*-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 subgrouping of *G. intestinalis* assemblage A into two subassemblages 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 *Tru9* I can provide a comple-

mentary, 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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