Minireview

Technical notes for the genotyping of *Giardia intestinalis*

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Abstract

The importance of molecular epidemiological techniques to the study of *Giardia intestinalis* (syn. *G. duodenalis, G. lamblia*) derives from the considerable complexity in its genetic diversity, host specificities and routes of transmission between humans and other mammalians. Recent advances in PCR-based genotyping methods for *G. intestinalis* have allowed us to characterize the isolates of this protozoan pathogen using multiple gene loci; however, many factors, such as high GC contents of genomic DNA sequence and extremely high micro-heterogeneities of genes within this species, still make it difficult to obtain PCR amplicons from target genes of this organism. Many attempts have been made to overcome these difficulties, including the use of additives in the PCR reaction mixture, and degenerated primers or inosine-introduced primers. Together with these adjustments, the careful selection of target areas and primers could improve our performance in genotyping this complex pathogen.

Key words: Giardia intestinalis; Molecular epidemiology; Genotyping; Glutamate dehydrogenase; Small subunit rRNA; Triose phosphate isomerase; Elongation factor 1-α.

1. Introduction

Recent advances in molecular epidemiological techniques applied to the study of *Giardia intestinalis* (syn. *G. duodenalis, G. lamblia*) have furthered our understanding of its genotypes. Phylogenetic analysis has shown this species has seven different genotypes (assemblages A to G) by means of analyses with various gene loci, such as the triose phosphate isomerase (TPI, Table 1), glutamate dehydrogenase (GDH, Table 2), small subunit ribosomal RNA (18SrRNA, Table 3) and elongation factor 1-α (EF1-α, Table 4) genes. Although it is still unclear how this intra-species diversity was produced, and even whether it is related to host specificity and the pathogenicity of *G. intestinalis*, it is clear that the only way to answer these remaining questions depends on the techniques of molecular epidemiological analyses. In this review, in an attempt to overcome the technical difficulties involved, we summarize the current status of analyses by focusing on PCR-amplicons obtained from various gene loci.

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2. Purification and concentration of cysts from samples

Since giardial cysts are excreted in the stools of human hosts, a purification step before DNA extraction of *Giardia* is desirable. In fecal samples from asymptomatic immunocompetent individuals in particular, the number of cysts excreted is expected to be low, a concentration step should be carried out whether *Giardia* is detectable or not. In most cases, this step is performed according to the density gradient centrifugation method using sucrose, glucose or other salts, i.e. zinc sulfate, sodium nitrate, CsCl and NaCl [1, 2, 4, 6, 7, 8, 10, 17, 22, 24, 25, 26 31, 36]. Alternative methods include an ethyl acetate concentration procedure [1, 25], gel filtration chromatography [1], and the use of anti-*Giardia*

magnetic beads, which often used to concentrate cysts from environmental water samples [8,10,11,18,19,20].

Table 1: Primers for TPI gene

Name	Sequence 5' - 3'	Acc. No.*	Positio n*	Ref.
A-for	GGAGACCGACGAGCAAAGC (F)	L02120	839-857	1
A-rev	CTTGCCAAGCGCCTCAA (R)	L02120	970-986	1
B-for	AATAGCAGCACARAACGTGTATCTG (F)	L02116	725-749	1
B-rev	CCCATGTCCAGCAGCATCT (R)	L02116	787-806	1
TPIA-F	CGAGACAAGTGTTGAGATGC (F)	L02120	758-777	1,8,16, 32
TPIA-IF	CCAAGAAGGCTAAGCGTGC (F)	L02120	859-877	8, 16
TPIA4IR	GCCACATGCCTATGTACGGG (R)	L02120	1291-310	8
TPIA-R	GGTCAAGAGCTTACAACACG (R)	L02120	1315-34	1,16, 32
TPIB-F	GTTGCTCCCTCCTTTGTGC (F)	L02116	663-681	1,8,16, 32
TPIB-IF	GCACAGAACGTGTATCTGG (F)	L02116	732-750	8, 16
TPIB-R	CTCTGCTCATTGGTCTCGC (R)	L02116	853-871	1,8, 16
TPIB4R	GGCCTTGCGTTCATCCAGG (R)	L02116	935-953	8
TPIBR2	AGGCAATTACAACGTTCTCCCA (R)	L02116	1026-47	32
AL3543	AAATIATGCCTGCTCGTCG (F)	L02116	538-556	3, 14
AL3546	CAAACCTTITCCGCAAACC (R)	L02116	1129-47	3, 14
AL3544	CCCTTCATCGGIGGTAACTT (F)	L02116	558-577	3, 14
AL3545	GTGGCCACCACICCCGTGCC (R)	L02116	1068-87	3, 14
TPIGENF	ATCGGYGGTAAYTTYAARTG (F)	L02116	564-583	5
TPI16F	CCCTTCATCGGYGGTAAC (F)	L02116	558-575	5
TPI533R	CCCGTGCCRATRGACCACAC (R)	L02116	1056-75	5
TPI572R	ACRTGGACYTCCTCYGCYTGCTC (R)	L02116	1092-114	5
TPIGENR	CACTGGCCAAGYTTYTCRCA (R)	L02116	1206-25	5
RTTPI F	ATYAAGAGCCACGTRGCGKC (F)	L02116	603-622	36
RTTPI R	CCATGATTCTRCGYCTTTCAG (R)	L02116	832-852	36
7493	GCAGAATGTGTACCTAGAGGGG (F)	L02120	719-740	26
5945	TAGTCTCCGAGCTCCTTCTGG (R)	L02120	1509-29	26
4131	ATGCCTGCTCGTCGCCCCTTC (F)	L02120	528-548	26
4130	CACTGGCCAAGCTTCTCGCAG (R)	L02120	1190-1210	26

*: Accession numbers and the primers position are from the DDBJ database and may not correlate with each specific reference.
(F): Foreword primer (R): Reverse primer

3. Extraction of DNA from cysts

While there are no specific difficulties with the DNA extraction of *Giardia* cysts, the robust cyst wall should be destructed before extraction. This destruction can be achieved after several freeze and thaw cycles with or without the combined use of zirconia or IMS beads [3, 6, 7, 8, 9, 11, 16, 19, 20]. For the DNA extraction protocol, phenol-chloroform extraction has been carried out [1, 2, 3, 11, 13, 14, 18, 19, 20, 22, 24, 25, 32] and various commercial kits have also been used [6, 8, 9, 10, 17].

Table 2: Primers for GDH gene

Name	Sequence 5' - 3'	Acc. No.*	Position*	Ref.
GDHeF	TCAACGTYAAYCGYGGYTTCCGT (F)	M84604	435-457	2
GDHiF	CAGTACAACTCYGCTCTCGG (F)	M84604	461-480	1, 2
GDHiR	GTTRTCCTTGCACATCTCC (R)	M84604	874-892	1, 2
GDH 1	ATCTTCGAGAGGATGCTTGAG (F)	M84604	362-382	20,31,34
GDH 3	TGTCCTTGCACATCTCCTCCA (R)	M84604	870-890	18
GDH 4	AGTACGCGACGCTGGGATACT (R)	M84604	1110-30	20,31,34
GDH 1F	AGGATGCTTGAGCCGGAGCG (F)	M84604	371-390	10,
GDH 4 R	GGATACTTNTCCYTGAACTC (R)	M84604	1097-116	10,
GDH F 3	TCCACCCCTCTGTCAACCTTTC (F)	U60983	107-128	10, 31
GDH B 5	AATGTCGCCAGCAGGAACG (R)	U60983	306-324	10, 31
578	GAGAGGATCCTTGARCCNGAGCG CGTNATC (F)	M84604	368-397	5, 15, 29
579	ACCTTCTAGAANCCNGCDATGTT NGCGCC (R)	M84604	1511-39	5, 15, 29
862	AGTACGCGACGCTGGGATACT (R)	M84604	1110-30	5, 15, 29
913	ATGACCGAGCT(T/C)CAGAGGC (F)	M84604	665-683	5, 15, 29
914	TGAACTCGTTCCTNAGGCG (R)	M84604	767-785	5, 15, 29
GAGDH 500	GAGATGTGCAAGGAYAAC (F)	M84604	875-892	5
GDHF	CCGCTTCCACCCCTCTGTCAA (F)	M84604	499-519	32
GDHR	CCTTGCACATCTCCTCCAGGAA (R)	M84604	866-887	32

*: Accession numbers and the primers position are from the DDBJ database and may not correlate with each specific reference.
(F): Foreword primer (R): Reverse primer

4. PCR amplification

The difficulties in obtaining PCR amplicons from target genes of this organism are well known. The primary reasons for this are the high GC contents of the genomic DNA sequence, and the extremely high micro-heterogeneities of genes within the population of *G. intestinalis*. To overcome these problems, PCR procedures using giardial genomic DNA as templates have been modified. Various additives used in PCR reaction mixtures, such as 5% [5, 15, 18, 27] or 10% [5] dimethyl sulfoxide (DMSO), Triton X-100 and gelatin [4, 5, 17, 27, 30, 36], and Taq Extender [4, 6, 30], have been thought to improve the linearity of the GC-rich template and increase the amplification efficacy of PCR. In some cases, a higher annealing temperature more than the optimum melting temerature of primers appeared effective in improving results (our observed data, unpublished). Some commercial GC-rich PCR system kits are also available [12, 14]. The GC-rich templates

could also affect the DNA sequencing protocols, and some researchers have reported modifications by the addition of 5 or 6% DMSO in the DNA sequence reaction mixture [17, 30].

Table 3: Primers for 18SrRNA gene

Name	Sequence 5' - 3'	Acc. No.*	Position*	Ref.
18S-1	TCCGGTCGATCCTGCCGGA (F)	M54878	3-21	18, 21, 33
18S-1(A)	GGTCGATCCTGCCGGAGCG (F)	M54878	6-24	21
18S-1(B)	GGTCGATCCTGCCGGAATC (F)	AF473852	6-24	21
18S-A	GCTCTCCGGAGTCGAAC (R)	M54878	285-301	18, 21, 33
18S-K	TGGCGGCGGGGGGCCTTC (R)	M54878	511-528	21
R39	CCCGGGATCCAAGCTTGATCCT TCTGCAGGTTCACCTAC	-	1425-45 **	21
18S H_a	GCCGGCTTGGCGGGTCG (R)	Z17210	1180-96	33
AL4303	ATCCGGTCGATCCTGCCG (F)	M54878	2-19	13, 14,
AL4305	AGTCGAACCCTGATCCT (R)	M54878	276-292	13, 14
AL4304	CGGTCGATCCTGCCGGA (F)	M54878	5-21	13, 14
AL4306	ACCCTGATCCTCCGCC (R)	M54878	271-286	13, 14
Gia2029	AAGTGTGGTGCAGACGGACTC (F)	-161 - -140 at 5` end		6
Gia2150c	CTGCTGCCGTCCTTGGATGT (R)	M54878	322-341	6
RH11	CATCCGGTCGATCCTGCC (F)	M54878	1-18	7, 17, 27, 28, 30, 35
RH4	AGTCGAACCCTGATTCTCCGC-CAGG (R)	M54878	268-292	7, 17, 27, 28, 30, 35
Gia-N	GTGATGCCCCGGAAGCCCG (R)	M54878	212-230	28
RM3`	CAGGTTCACCTACGGATACC (R)	M54878	1419-38	4
GiarF	GACGCTCTCCCCAAGGAC (F)	M54878	26-43	35, 36
GiarR	CTGCGTCACGCTGCTCG (R)	M54878	183-199	35, 36
G18S2	TCCGGTYGATTCTGCC (F)	M54878	3-18	5
G18S3	CTGGAATTACCGCGGCTGCT (R)	M54878	446-466	5

*: Accession numbers and the primers position are from the DDBJ database and may not correlate with each specific reference.

**: As indicated in the original reference

(F): Foreword primer (R): Reverse primer

5. Primer selection

For the second main problem, concerning extremely high micro-heterogeneities of genes within the population of *G. intestinalis*, the best way forward might be the careful selection of primers for the PCR protocol. Most primers used in giardial genotyping were of degenerated conformations and some used inosine as a wild-card-like nucleotide, as shown in Tables 1, 2, 3 and 4. To allow for easy selection, use and reference to the original main article, we show most published sets of primers targeting TPI, GDH, 18SrRNA and EF1-α genes in Tables 1, 2, 3 and 4, respectively. To help with the selection of an appropriate primer set for PCR, the primer targeting sequence position on the reference gene with the DDBJ accession number is shown in these tables. The primers targeting specific genes in each species or genotype could be helpful in the detection and differentiation of the different genotypes, especially when mixed infection is suspected. The use of different primers at different positions of a same target gene offers a very good opportunity to use a number of alternatives in the nested PCR, which could be of great value in the confirmation of DNA sequences.

Table 4: Primers for EF1-α gene

Name	Sequence 5' - 3'	Acc. No.*	Position*	Ref.
GLongF	GCTCSTTCAAGTACGCGTGG (F)	D14342	95-114	4, 5
EF1AR	AGCTCYTCGTGRTGCATYTC (R)	D14342	784-803	4, 5
GLONGR	GCATCTCGACGGATTCSACC (R)	D14342	771-791	5
RTefαF	GCCGAGGAGTTCGACTACATC (F)	D14342	496-516	36
RTefαR	GACGCCSGAGATCTTGTAGAC (R)	D14342	664-684	36

*: Accession numbers and the primers position are from the DDBJ database and may not correlate with each specific reference.

(F): Foreword primer (R): Reverse primer

6. Concluding remarks

The genotype construction of the giardial population appears to have extremely high diversity, and thus future molecular epidemiological study of *Giardia* spp. needs a careful research designing. Assemblage analysis should be undertaken on the basis of at least two gene loci according to the previous reference information, where one of these loci should target a universal coding region such as 18SrRNA or EF1-α, and another should be suitable for further sub-typing analysis such as TPI or GDH [23]. Accurate and detailed molecular epidemiological data will directly contribute to a better understanding of the intra-species diversity of *G. intestinalis*. Such data will also provide novel insight into the evolution and distribution of assemblages, as well as the relationships between assemblages and host specificity and pathogenicity of *G. intestinalis*.

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Minireview

Genotyping of *Cryptosporidium* species: current status and future direction

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Abstract

Molecular epidemiology has provided novel insights into the genetic diversity of the genus *Cryptosporidium*, one of the major causative protozoan parasites of diarrhea in humans and various animals worldwide. Genetic analysis using various target loci on genomic DNA has revealed that *Cryptosporidium* consists of at least 15 species and various genotypes with different host specificities and pathogenicities. Although it is currently unclear how such diversity was produced and what physiological significance is related to these differences, consideration of the evolutional history together with phylogenetic analyses of gene sequences will likely provide useful insights for future study of the heterogeneity of this parasite.

Key words: *Cryptosporidium*; Molecular epidemiology; Genotyping; Phylogenic analysis; Small subunit ribosomal RNA; *Cryptosporidium* oocyst wall protein; 70 kDa Heat shock protein; Actin; Polythreonine.

1. Introduction

Due to advances in molecular epidemiological techniques, 15 species of *Cryptosporidium* are thus far regarded as valid: *C. muris* in rodents; *C. andersoni* and *C. bovis* in cattle; *C. parvum* in ruminants and humans; *C. wrairi* in guinea pigs; *C. hominis* in humans; *C. meleagridis*, *C. baileyi* and *C. galli* in birds; *C. serpentis* and *C. aurophilum* in snakes and lizards; *C. molnari* in fish; *C. felis* in cats; *C. canis* in dogs; and *C. suis* in pigs. At least 7 species (*C. hominis, C. parvum, C. meleagridis, C. felis, C. canis, C. suis and C. muris*) and 2 genotypes (monkey and cervine) of *Cryptosporidium* have been reported from human clinical cases (reviewed in [2]).

The current tools for genotyping of *Cryptosporidium* spp. involve the combination of polymerase chain reaction (PCR) targeting various gene loci, e.g., 18S ribosomal RNA (18SrRNA), *Cryptosporidium* oocyst wall protein (COWP), 70 kDa heat shock protein (HSP70), actin and polythreonine (Poly-T) genes, with restriction fragment length polymorphism (RFLP) or direct sequencing. These methods have been widely used because of their high sensitivity for detecting the genomic DNA of this parasite and the existence of valuable research data reported from a wide range of organisms, e.g., pets, livestock, wild animals and humans.

This review summarizes the essential aspects of the molecular epidemiological approach to *Cryptosporidium* spp. and also the availability of primers and reference sequences held in GenBank of each target gene locus.

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2. Significance of reference DNA sequences in genetic analysis

As shown in Tables 1 to 4 and Fig. 1, numerous molecular techniques have been developed for the differentiation of *Cryptosporidium* genotypes and species. Although various RFLP methods have been used in the genotyping of *Cryptosporidium*, the reference sequences, which are available from GenBank, are indispensable in its molecular epidemiological analysis. The RFLP method is an epidemiologically important method that determines the genotype of isolates; however, the results seem to lack applicability

to the evaluation of the origin or biological significance of heterogeneity in *Cryptosporidium*. That is to say, only an accumulation of reference sequence data of various gene loci in the genotyping of this parasite is considered to reveal its detailed population structure, geographical distribution and evolutional process.

This review therefore focuses on the use of DNA sequences and the PCR method to obtain amplicons from genomic DNA of *Cryptosporidium* isolates.

3. Various target loci for the genotyping of *Cryptosporidium* spp.

The genotyping methods currently used differ with respect to the genetic locus targeted.

Table 1. PCRprimers targeting 18S rRNA gene

Method	Name	Primers	Amplicon size (bp)	Ref.
PCR	SSU-F1	AACCTGGTTGATCCTGCCAGTAGTC	1,750	5
	SSU-R1	TGATCCTTCTGCAGGTTCACCTACG		
Direct sequence	18SiF	AGTGACAAGAAATAACAATACAGG	300	4
PCR-SSCP	18SiR	CCTGCTTTAAGCACTCTAATTTTC		
nested PCR-RFLP Direct sequences	SSU-F2	TTCTAGAGCTAATACATGCG	1,325	5, 13
	SSU-R2	CCCATTTCCTTCGAAACAGGA		
	SSU-F3	GGAAGGGTTGTATTTATTAGATAAAG	820	
	SSU-R3	AAGGAGTAAGGAACAACCTCCA		
nested PCR-RFLP	ExCry1	GCCAGTAGTCATATGCTTGTCTC	840	8
	ExCry2	ACTGTTAAATAGAAATGCCCCC		
	NesCry3	GCGAAAAAACTCGACTTTATGGAAGG(	590	
	NesCry4	GGAGTATTCAAGGCATATGCCTGC		
nested PCR Direct sequence	18SiCF2	GACATATCATTCAAGTTTCTGACC	760	12
	18SiCR2	CTGAAGGAGTAAGGAACAACC		
	18SiCF1	CCTATCAGCTTTAGACGGTAGG	587	
	18SiCR1	TCTAAGAATTTCACCTCTGACTG		
PCR	CPB-DIAGF	AAGCTCGTAGTTGGATTTCTG	435	3
	CPB-DIAGR	TAAGGTGCTGAAGGAGTAAGG		
nested-PCR RFLP	N-DIAGF2	CAATTGGAGGGCAAGTCTGGTGCCAG(	667	10
	N-DIAGR2	CCTTCCTATGTCTGGACCTGGTGAGT		
	CPB-DIAGF	AAGCTCGTAGTTGGATTTCTG	435	
	CPB-DIAGR	TAAGGTGCTGAAGGAGTAAGG		

3.1. 18SrRNA gene: The optimum method of genotyping *Cryptosporidium* spp. has involved the use of the 18SrRNA gene to date, since its locus consists of five copies on the genome, which achieves high detection sensitivity. The locus was originally analyzed by a PCR-RFLP method using oocysts from environmental samples [3], and subsequent detailed studies revealed sequences of the locus for all 15 species and for over 30 genotypes, as indicated in Fig. 1 (A) [1, 5, 6, 8, 10, 11, 12]. For primer sets (Table 1), the most commonly used combination is SSU-F1/R1 with SSU-F2/R2 or SSU-F3/R3 [13], while for nested primers sets, ExCry1/2 and NesCry3/4 can detect *Cryptosporidium* at the single-oocyst level [8]. With the latter amplicon, RFLP analysis using VspI and DraII can differentiate *C. parvum* isolates from *C. baileyi* and *C. serpentis* [8], although this RFLP analysis can not classify all human pathogenic *Cryptosporidium* species [9]. The nested primer sets of N-DIAGF2/GR2 and CPB-DIAGF/R, however, which were designed based on a CPB-DIAGF/R single primer set [3], can identify almost all species and genotypes by nested PCR-RFLP using 5 or fewer oocysts [10].

Table 2. PCR primers targeting COWP gene

Method	Name	Primers	Amplicon size (bp)	Ref.
PCR-RFLP Direct sequence	cry15	GTAGATAATGGAAGAGATTGTG	580	14
	cry9	GGACTGAAATACAGGCATTATCTTG		
nested PCR-RFLP	BCOWPF	ACCGCTTCTCAACAACCATCTTGTCCTC	769	17
	BCOWPR	CGCACCTGTTCCCACTCAATGTAAACCC		
nested PCR-RFLP Direct sequence	oocry 3	AGATTAACAGAATGCCCACCAGGTA	923	15
	oocry 4	CCATGATGATGTCCTGGATTTTGTA		
	oocry 1	CCTGGATATCTCGACAAT	640	
	oocry 2	GCGAACTAATCGATCTCTCT		
nested PCR-RFLP Direct sequence	PCOWPF	GTGTACAGTCTGATACTGCACC	435	18
		GTGTACAGTCTGACACTGCACC		
		GTGTTCAATCAGATACTGCCCC		
		GCGTACAGTCAGATACTGCTCC		
		GTGTTCAATCAGACACAGCTCC		
		GTGTACAATCAGATACCGCTCC		
	PCOWPR	GGACATACTGGTTGTGTTG		
		GGACATATAGGTTGAGTTG		
		GGACATACTGGTTGAGTTG		
		GGGCATACAGGTTGTGTTG		
		GGGCAGACAGGTTGAGTTG		
		GGACAAACAGGTTGAGTTG		
	PCOWPIF	CCTCCCAACCCTGAATGTCC	341	
		CCACCTAACCCAGAATGTCC		
		CCACCAAACCCAGAATGTCC		
		CCTCCTAATCCAGAATGTCC		
		CCACCAAATCCAGAATGTCC		
	PCOWPIR	GGGCACTCCTTAGCTGGAGC		
		GGGCATTCCTTAGCTGGAGC		
		GGACATTCTTTTGCAGGAGC		
		GGACATTCTTTCGCAGGAGC		
		GGGCATTCCTTTGCAGGAGC		
		GGGCACTCTTTTGCAGGAGC		

3.2. COWP gene: For primer sets (Table 2), COWP PCR was initially carried out using a Cry 9/15 primer set [14], and then improved to a more sensitive nested PCR method using a novel outer BCOWPF/ R primer set [17]. Oocry 3/4 and oocry 1/2 primer sets, which were also designed for a single-tube nested PCR method targeting the COWP gene, achieve high sensitivity in obtaining a PCR amplicon from 250fg *C. parvum* genomic DNA template [15]. However, neither the BCOWPF/R nor oocry 3/4 primer set can detect the genomic DNA templates of *C. felis*, *C. canis* or *C. suis*. In contrast, the Cry 9/15 primer set can amplify all 17 species and genotypes of *Cryptosporidium*, which are shown in Fig. 1 (B)[9, 14], although it should be noted that a comparatively low level PCR amplification was obtained from genomic DNA templates of *C. canis*, *C. felis*, *C. baileyi*, *C. muris*, *C. andersoni* and *C. serpentis*, possibly due to the sequence polymorphism of the primer region of Cry 9/15 [16]. To overcome this difficulty with low-level amplification of COWP genes, PCOWF/R and PCOWIF/IR primer sets were designed, consisting of 6 and 5 different primer sets, respectively [18]. For COWP-based genotyping, RFLP analysis using Cry 9/15 can not differentiate the isolates of: (i) *C. muris*, *C. andersoni*, and *C. serpentis*; (ii) *C. parvum* ferret genotype and *C. wrairi*; (iii) *C. parvum* bovine and mouse genotypes; or (iv) *C. parvum* human and monkey genotypes [16]. However, a Cry 9/15 primer set is considered useful for the genotyping of human clinical isolates of *Cryptosporidium* spp. even with the RFLP method [13].

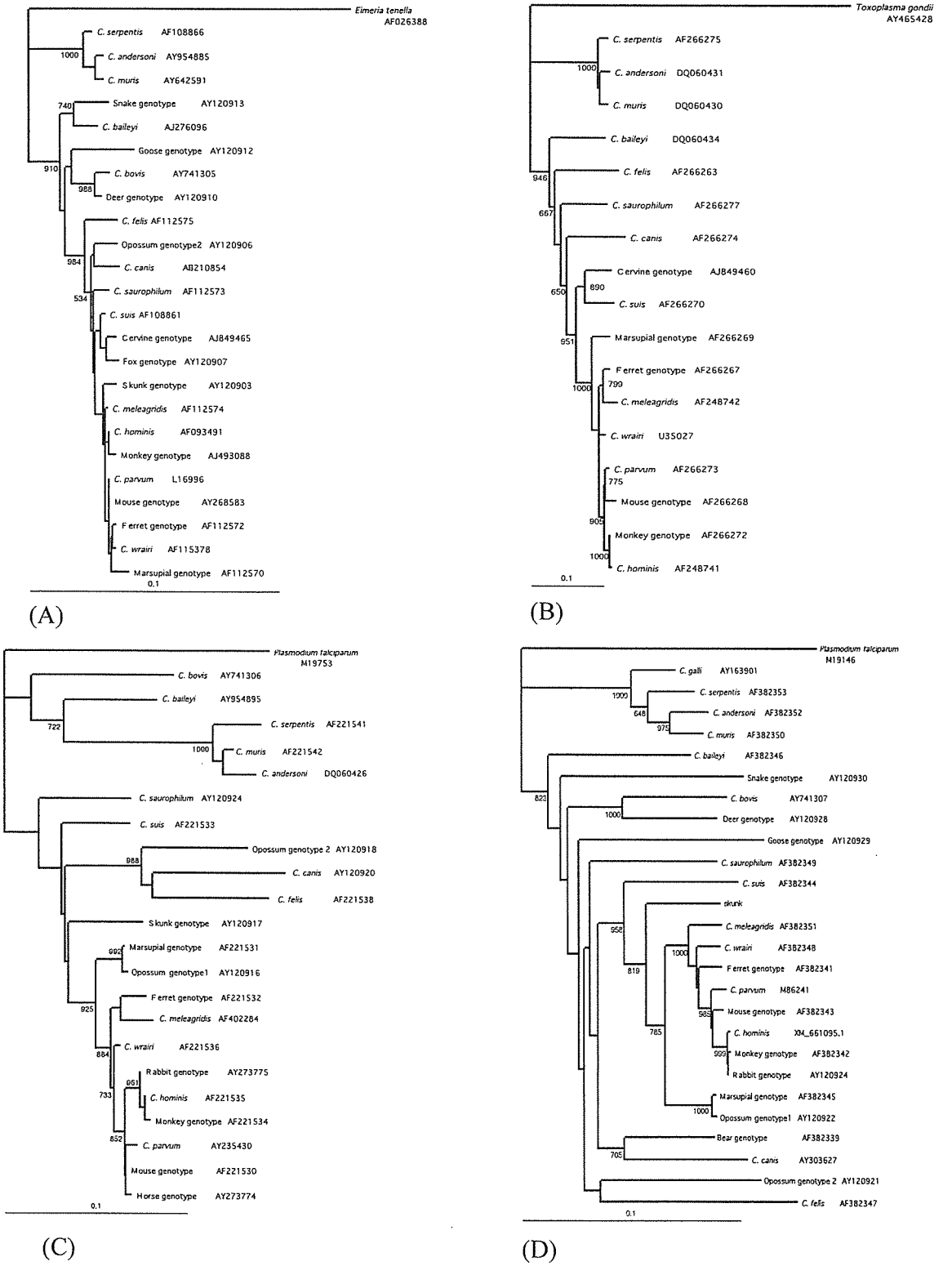

Fig. 1 Phylogenetic relationship among *Cryptosporidium* species and genotypes inferred by NJ analysis (A): 18SrRNA gene, (B): COWP gene, (C): HSP70 gene and (D): actin gene. As an out-grope, *Eimeria tenella*, *Toxoplasma gondii* and *Plasmodium falciparum*, which are coccidian parasites, were used respectively. Values on branches are bootstrapping using 1,000 replicates. Numbers, shown after names of species or genotypes, indicate the GenBank accession numbers.

Table 3. PCR primers targeting HSP70 gene

Method	Name	Primers	Amplicon size (bp)	Ref.
Direct sequence	HSP-F1	ATGTCTGAAGGTCCAGCTATTGGTATTGA	2,010	20
	HSP-R1	TTAGTCGACCTCTTCAACAGTTGG		
	HSP-F2	TA/CTTCATG/CTGTTGGTGTATGGAGAAA	1,950	
	HSP-R2	CAACAGTTGGACCATTAGATCC		
PCR-RFLP Direct sequence	Cshp1	AGCAATCCTCTGCCGTACAGG	580	21
	Cshp4	AAGAGCATCCTTGATCTTCT		
Direct sequence	Cshp2386F	CTGTTGCTTATGGTGCTGCTG	300	19
	Cshp2672R	CCTCTTGGTGCTGGTGGAATA		
Direct sequence	HSPF4	GGTGGTGGTACTTTTGATGTATC	450	22
	HSPR4	GCCTGAACCTTTGGAATACG		
	HSPF3	GCTGSTGATACTCACTTGGGTGG	325	
	HSPR3	CTCTTGTCCATACCAGCATCC		

*3.3. **HSP70 gene:*** For primer sets (Table 3), the Cshp2386F/2672R primer set was initially designed for reverse-transcription PCR to evaluate the viability of cryptosporidal oocysts in environmental water samples [19]. In order to use HSP70 gene-targeting PCR as a diagnostic tool of human cryptosporidiosis, HSP-F1/R1 and HSP-F2/R2 primer sets were developed, which can amplify the HSP70 gene locus of most apicomplexian parasites, and the sets have been used as a standard nested-PCR method for the HSP70 gene [20]. Due to the universal characteristic of the primer sets, reference sequence information from various organisms has accumulated, as shown in Fig. 1 (C); however, the amplicon size (1,950 bp) is too long for amplification of genomic DNA of isolates in clinical laboratories. The development of HSPF4/R4 and HSPF3/R3 nested PCR primer sets improved the product size to 325bp [22]. Also and the Cshp1/4 primer can also amplify a 580 bp product from the genomic DNA of only one oocyst [21]. In exchange for achieving high sensitivity, however, Cshp1/4 can not detect the DNA of *C. suis*, *C. felis*, *C. muris* or *C. canis* [9].

Table 4. PCR primers targeting actin and poly-T genes

Target Gene	Method	Name	Primers	Amplicon size (bp)	Ref.
Actin	Direct sequence	Act-F1	ATGA/GGA/TGAAGAAGA/TAA/GC/TA/TCAAGC	1,095	23
		Act-R1	AGAAG/ACAC/TTTTCTGTGT/GACAAT		
		Act-F2	CAAGCA/TTTG/AGTTGTTGAT/CAA	1,096	
		Act-R2	TTTCTGTGT/GACAATA/TG/CA/TTGG		
poly-T	PCR-RFLP	cry 44	CTCTTAATCCAATCATTACAAC	520	24
		cry 37	CAGCAAGATATGAATACCG		

*3.4. **Actin and Poly-T genes:*** The actin and Poly-T loci have been examined because of the possibility that phylogenetic analysis results using only the 18SrRNA locus may not accurately reflect the evolutional relationship of *Cryptosporidium* spp. [23, 24]. Widespread use of the Act-F1/R1 and Act-F2/R2 nested PCR primers (Table 4) targeting the actin gene have produced reference sequence information for various species and genotypes, as shown in Fig. 1 (D). In Poly-T genes, the length polymorphism of multiple polythreonine motif was expected and analyzed by Cry37/44 primer set, which could not detect *C. muris* and *C. serpentis* [24, 7].

*3.5. **Other gene loci:*** Other gene loci including thrombospondin-related adhesive protein 1 (TRAP-C1) [25], TRAP-C2 [26] and beta-tubulin [27] have been used to differentiate certain species and for the genotyping of *Cryptosporidium*. Currently more detailed analyses for sub-genotyping are available, utilizing extra-chromosomal double strand rRNA [28] and 60 kDa glycoprotein (GP60) gene. The results of GP60 gene sub-genotyping analysis, in particular, have provided new insight into the subtypes of *C. hominis* (Ia, Ib, Ic, Id, Ie) and *C. parvum* (IIa, IIb, IIc, IId, IIe, IIf) [29, 30, 31, 32, 33].

4. Concluding remarks

This review demonstrates that reference sequences of various loci of *Cryptosporidium* spp. from various hosts, ranging from mammalians to reptiles, have been fully reported in excellent previous works. Thus, future genotyping studies should undertake accurate analysis using these previous data, and any novel sequence data should be deposited in public databases to contribute to further advances in this field. Even though extensive molecular epidemiological data is now available, the evolutional process and the biological significance of the heterogeneity in *Cryptosporidium* remain to be elucidated. Therefore, with regard to the future perspective of the research on *Cryptosporidium*, the evolutional and physiological significance of intra-species heterogeneity are likely to be important topics for researchers in the next decade.

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The running head: Entamoeba histolytica variant from primates

PROFILES OF A PATHOGENIC ENTAMOEBA HISTOLYTICA-LIKE VARIANT WITH VARIATIONS IN THE NUCLEOTIDE SEQUENCE OF THE SMALL SUBUNIT RIBOSOMAL RNA ISOLATED FROM A PRIMATE (DE BRAZZA'S GUENON)

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Abstract: A pathogenic Entamoeba histolytica-like variant (JSK2004) with genetic variations and a novel isoenzyme pattern was isolated from a De Brazza's guenon. A homology of 99.1% was found between the clones of E. histolytica (HM-1:IMSS) and JSK2004 in the 1893 nucleotide bases of the small subunit rRNA (SSU-rRNA) gene. The DNA of the pathogenic amoeba species was also extracted from 2 sterile liver abscesses during the autopsies of an Abyssinian colobus and a Geoffroy's spider monkey in the same institution that JSK2004 was isolated, and the homology of the nucleotide sequences in the SSU-rRNA gene of the DNAs were identical to that of JSK2004.

Key words: Entamoeba histolytica-like variant, pathogenic isolate, Primate, SSU-rRNA gene, De Brazza's guenon.

BRIEF COMMUNICATION

Entamoeba histolytica (pathogenic) and Entamoeba dispar (nonpathogenic) are parasitic amoebic species in humans and nonhuman primates and they show high genetic similarity.[2, 12] Since it is difficult to morphologically differentiate the latter from the former, the detection of species-specific hexokinase (HK) and phosphoglucomutase (PGM) isoenzyme bands,[9] the detection of proteins by monoclonal antibodies,[6] and the detection of DNAs by a polymerase chain reaction (PCR)[4, 10] have been used for identification. Previously, a pathogenic E. histolytica variant was detected with isoenzyme bands characteristic of both E. histolytica PGM and E. dispar HK. This exceptional isoenzyme pattern [zymodeme XIII (Z-XIII)] was detected in human cases in South Africa and Tanzania; however, the genetic profile of this variant has not been described.[9] In this study, we isolated a pathogenic E. histolytica-like strain (JSK2004) from a De Brazza's guenon (Cercopithecus neglectus). This strain did not satisfy the criteria for zymodeme classification; however, the isoenzyme bands were characteristic of both E. histolytica HK and E. dispar PGM that were inverse patterns of Z-XIII.

Prior to the isolation of JSK2004, the DNA of the pathogenic amoeba species was extracted from 2 sterile liver abscesses during the autopsies of an Abyssinian colobus (Colobus guereza) and a Geoffroy's spider monkey (Ateles geoffroyi). DNA was also extracted from the feces, including cysts, of a De Brazza's guenon without distinct symptoms and it was identified as that

of E. histolytica by using the E. histolytica II kit (TechLab, VA, USA); JSK2004 was isolated from the same individual. The abovementioned three primate species were born and bred for several generations in Japan. The infection source could not be definitely identified. The specimens were subjected to PCR and multiplex PCR by using 2 primer sets targeting the 30-kDa proteins[10] and the small subunit rRNA (SSU-rRNA) genes[4] of E. histolytica and E. dispar, respectively. The expected 101-bp fragments of the E. histolytica gene were produced by PCR. However, no fragment was produced by the multiplex PCR (data not shown).

A JSK2004 axenic culture was established in TYI-S-33 medium;[1] subsequently, 4 clones (JSK2004 cl1 to cl4) were obtained by the classical methods of Diamond and Gillin.[1] Each clone was confirmed to possess the same genetic polymorphism[5] and zymodeme[9] profiles. We sequenced 1893 bases of the SSU-rRNA gene of 1 clone (JSK2004 cl2). The gene was PCR-amplified using the Entamoeba species-specific primer set [Entam1 (forward: 5′-GTT GAT CCT GCC AGT ATT ATA TG-3′) and Entam2 (reverse: 5′-CAC TAT TGG AGC TGG AAT TAC-3′)] (11) and 2 primer sets [Ent2F (forward: 5′-GTA ATT CCA GCT CCA ATA GTG-3′) and Ent2R (reverse: 5′-ACA CCA CTT ACT ATC CTT AAT-3′), Ent3F (forward: 5′-GTT ATC TAA TTT CGG TTA GAC-3′) and Ent3R (reverse: 5′-ATC CTT CCG CAG GTT CAC CTA-3′)] that were designed from the SSU-rRNA gene sequence of an E. histolytica reference strain [HM-1:IMSS cl6 (HM-1)] (GenBank accession no. X64142). The amplification was performed in

a reaction mixture (50 μl) containing 100 ng DNA, 1.0 U LATaqDNA polymerase (Takara Bio Inc., Shiga, Japan), 0.4 μM of each primer, and 0.25 mM dNTPs. The following cycling parameters were used: (i) Taq activation at 94°C for 3 min; (ii) 35 cycles of denaturation at 94°C for 40 s, annealing at 51°C for 40 s, and extension at 72°C for 1 min; and (iii) extension at 72°C for 5 min. The PCR products were sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, CA, USA) on an ABI PRISM 310 Genetic Analyzer.

A comparison of the SSU-rRNA sequences of JSK2004 cl2, HM-1, and SAW1734R clAR (SAW1734; an E. dispar reference strain; GenBank accession no. Z49256) indicated that 14 nucleotide bases of the SSU-rRNA sequence of JSK2004 cl2 differed from those of HM-1, and of these 14 bases, 7 were similar to those of SAW1734 (Fig. 1). Similarly, the SSU-rRNA gene sequences of JSK2004 cl1, cl3, and cl4 corresponded to that of JSK2004 cl2. The SSU-rRNA sequences of the 3 other E. histolytica axenic strains with different zymodemes [Z-IIα (SAW1627), Z-XIV (SAW755R clB), and Z-XIX (KU-2)][5] and 2 E. dispar axenic strains (CYNO 09:TPC and AS16IR) (Z-I)[8] corresponded to those of HM-1 (Z-II) and SAW1734 (Z-I), respectively.

The total DNA from the liver abscess of the Abyssinian colobus and Geoffroy's spider monkey were PCR-amplified using 2 primer sets [Ent2F and Ent2R] and [Ent4F (forward:

5′-ATG GCC AAT TTA TTT AAA TGA-3′) and Ent4R (reverse: 5′-CAG ATT AAG AAA CAA TGC TTC-3′) designed from the SSU-rRNA gene sequence of JSK2004 cl2] for identifying the E. histolytica-like variant (JSK2004). The PCR was performed as follows: 5 min at 94°C; 35 cycles of 40 s at 94°C, 40 s at 55°C, 1 min at 72°C; and a final step of 5 min at 72°C. The PCR products from the total DNA from the liver abscess of an Abyssinian colobus and Geoffroy's spider monkey was obtained using 2 primer sets [(Ent2F and Ent2R) and (Ent4F and Ent4R)]. The authenticity of the PCR products was confirmed by nucleotide sequencing of the 878-bp and 1088-bp fragments, and the SSU-rRNA gene sequences corresponded to that of JSK2004 cl2 (Data not shown).

The isoenzyme patterns of 4 enzymes of the JSK2004 strain and its 4 clones were identical and novel, and showed the following bands: (i) malic enzyme (ME), band with the same mobility as those of E. histolytica and E. dispar; (ii) HK, fast-running double bands with the same mobility as those of E. histolytica HK; (iii) PGM, band (α band) of E. dispar-PGM type; and (iv) glucose phosphate isomerase (GPI), a previously unidentified band that corresponded to the γ band (Fig. 2).

The ability of the axenic-cultured JSK2004 to form liver abscesses and its infectious capacity in the large intestine were examined by inoculating 1×10^6 amoebae/head into the left hepatic lobes of female Syrian hamsters (3–4 wk old)[3] and the cecums of female C3H/HeJ mice (5–6 wk

old).[7] Liver abscess formation was confirmed in all the 3 examined hamsters; JSK2004 established persistent infection in 3 mice for more than 6 mo. The homologies of 1893 nucleotide bases of the SSU-RNA gene between JSK2004 cl2 and E. histolytica and E. dispar were 99.10% and 98.47%, respectively. Hence, the genetic homology between E. histolytica and E. dispar was 98.10%; JSK2004 cl2 is located at a position that is intermediate between the 2 species. The pathogenic E. histolytica-like variant (JSK2004) that is located at an intermediate position between E. histolytica and E. dispar and contains an E. dispar-PGM type isoenzyme and variations in the SSU-rRNA gene is expected to be a useful reference for phylogenetic studies.

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