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Multiple-subgenotype infections of *Giardia intestinalis* detected in Palestinian clinical cases using a subcloning approach

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ABSTRACT

To evaluate the geographic distribution of *Giardia intestinalis* genotypes in Nablus, West Bank, Palestine, a genotyping study was performed using clinical fecal samples. Microscopic examination confirmed that 8 of 69 (11.6%) samples were *G. intestinalis* positive, and subsequent genotyping analyses targeting the small-subunit ribosomal RNA (18S rRNA) and glutamate dehydrogenase (GDH) genes revealed the *G. intestinalis* genotypes within the 8 samples. Of these 8 samples, 6 were clustered with assemblage A-II and the remaining 2 samples were clustered with assemblage B by 18S rRNA gene analysis; however, direct sequencing of the GDH gene segments from the latter 2 samples showed a mixed infection profile. To assess those samples, we employed a subcloning approach and successfully isolated 6 independent assemblage B subgenotypes. These partial GDH gene sequences (393 bp) had 15 single-nucleotide polymorphisms, all of which were synonymous transition substitutions at the third nucleotide position of codons. From the results, we concluded that the highly polymorphic gene loci such as GDH gene locus might provide us an opportunity to obtain a detailed molecular data even from the samples with multiple-subgenotype mixed infections. Therefore, subcloning approach is recommended in genotyping studies, especially in those conducted in giardiasis-endemic areas, where the repeated and cumulative infections could be commonly expected.

1. Introduction

Giardia intestinalis (syn. *G. duodenalis* and *G. lamblia*) inhabits the small intestine, causing a wide range of symptoms and conditions, from asymptomatic to severe diarrhea with or without malabsorption and weight loss. In developing countries, the prevalence of giardiasis in patients with diarrhea has been reported to be approximately 20% (range, 5%–43%) [1]. According to the World Health Organization (WHO) estimates, the prevalence of symptomatic giardiasis is about 200 million cases worldwide with some 500,000 new infections each year [2]. Giardiasis is thought to be the most common community-derived disease leading to significant morbidity and mortality world-wide [3,4]. Person-to-person, zoonotic, water-borne, and food-borne transmissions can occur through the fecal–oral route after direct or indirect contact with the infective-stage cysts of the organism [3,5,6].

The presence of morphologically indistinguishable characteristics such as host specificity of *G. intestinalis* isolated from humans and various animals has led to the advanced use of genetic markers to analyze diversity at the genomic DNA level [7–10]. These molecular studies have efficiently discriminated the assemblages and revealed the complex genotypic structures within *G. intestinalis* populations [10,11]. At least 7 major assemblages, A–G [10], are considered valid in this species. The major genotypes of *G. intestinalis* isolated from humans have been reported as assemblages A and B, with 2 well-known subgenotypes in each, namely, subgenotypes A-I and A-II in assemblage A and subgenotypes B-III and B-IV in assemblage B [9,10,12]. These subgenotypes are used as references in many studies, and their detection in animal sources suggests the alarming possibility of zoonotic transmission [11,13]. Furthermore, detection of viable *G. intestinalis* cysts in fly has suggested that intense transmission could also occur indirectly [14]. The remaining genetic assemblages (C, D, E, F, and G) appear to be host-restricted to animals [4,10].

At present, the occurrence of mixed infections of *G. intestinalis* [15,16], the role of different assemblages and subgenotypes [15], genetic diversity [17], frequency of transmission [11,18], evolution [10], and clinical significance [19,20] remain debatable in genotyping studies. To address these issues, molecular epidemiological studies are required especially in endemic areas [4]; however, most molecular epidemiological studies have been conducted in developed countries [11,12,21,22].

In the present study, we collected *G. intestinalis* samples from Nablus, West Bank, Palestine, and performed subcloning analysis by using genomic DNA directly extracted from human fecal specimens to

Abbreviations: 18S rRNA, 18S small-subunit ribosomal RNA; GDH, glutamate dehydrogenase; SNPs, single-nucleotide polymorphisms; WHO, World Health Organization; PBS, phosphate-buffered saline; PCR, Polymerase chain reaction; dNTP, deoxynucleotide triphosphate; DMSO, dimethyl sulfoxide; DDBJ, DNA Data Bank of Japan; NJ, neighbor-joining; EMBL, European Molecular Biology Laboratory.

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Table 1
Genotyping results assessed in this study.

Sample name	Genotype
PalH1	Assemblage A
PalH2	Assemblage A-II
PalH3	Assemblage A
PalH4	Assemblage B[a]
PalH5	Assemblage A-II
PalH6	Assemblage A-II
PalH7	Assemblage A-II
PalH8	Assemblage B[a]

Genotypes of the PalH1 and PalH3 samples, which were identical to an assemblage A reference (AF199446) and those of the PalH4 and PalH8 samples, which were identical to an assemblage B reference (AF199447), were determined by 18S rRNA gene analysis. Assemblage A-II and multiple-subgenotype infections of assemblage B were determined by GDH gene analysis.

[a] Multiple-subgenotype infection of assemblage B.

evaluate the geographic distribution of the *G. intestinalis* genotypes and to better understand the occurrence of mixed-genotype infection in an endemic area.

2. Materials and methods

2.1. Fecal sample collection and microscopic examination

Sixty-nine fecal samples were obtained from patients who sought medical treatment for abdominal complaints during February and March 2006 in Nablus, West Bank, Palestine. All the samples were preserved in 2.5% (w/v) potassium dichromate at 4 °C and subsequently processed for cyst purification by using the sucrose centrifugal flotation method as described previously [23]. The presence of pathogenic intestinal protozoan parasites and the number of cysts were microscopically assessed with 600× magnification using some of the purified samples. The cyst count was semi-quantitatively showed as high (+++; multiple cysts in 1 view field), moderate (++; single cyst in 1 view field), and low (+; single cyst in multiple view fields). The purified cysts were stored at −20 °C in phosphate-buffered saline (PBS; pH 7.2) until further analysis. Each clinical fecal sample was collected after obtaining informed consent from the patients and providing them with a unique identification number on site in order to protect their personal information.

2.2. DNA extraction

After 3 cycles of freezing at −80 °C and thawing at 95 °C, genomic DNA was extracted from the cysts in PBS solution by using the QIAamp DNA Mini Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's instructions. The extracted genomic DNA was concentrated by ethanol precipitation and preserved as an aqueous solution at −20 °C until use.

2.3. Polymerase chain reaction (PCR) targeting the 18S rRNA gene locus

A partial DNA fragment of the 18S rRNA gene of *G. intestinalis* was amplified using previously described primers [10] (G18S2: 5′-TCCGGTYGATTCTGCC-3′ and G18S3: 5′-CTGGAATTACCGCGGCTGCT-3′) in 0.2-ml thin-walled PCR tubes on MyCycler (BioRad Laboratories, California, USA). PCR was carried out in a 20-μl reaction mixture containing 1 μl of the extracted *Giardia* DNA template, 1× GC buffer I, 0.4 μM of each primer, 0.5 mM of each deoxynucleotide triphosphate (dNTP), and 1 U of LA Taq polymerase (TaKaRa Bio Inc, Shiga, Japan), with 5% dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Osaka, Japan) as an additive. The cycling parameters were as follows: initial denaturation at 94 °C for 1 min; followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 50 s; and a final extension at 72 °C for 5 min. The PCR products were electrophoresed on 2% LO3 agarose (TaKaRa). The purified products were stained with ethidium bromide and then visualized on a UV transilluminator. The target fragments were then purified from the agarose gel by using the Quantum Prep™ Freeze 'N Squeeze DNA Gel Extraction Spin Columns (BioRad Laboratories) according to the manufacturer's instructions.

2.4. PCR targeting the GDH gene locus

A partial DNA fragment of the GDH gene of *G. intestinalis* was amplified using previously described seminested PCR primers [24] (GDHeF: 5′-TCAACGTYAAYCGYGGYTTCCGT-3′, GDHiF: 5′-CAGTAC-AACTCYGCTCTCGG-3′, and GDHiR: 5′-GTTRTCCTTGCACATCTCC-3′) in 0.2-ml thin-walled PCR tubes on MyCycler (BioRad Laboratories). Primary PCR was carried out in a 20-μl reaction mixture containing 1 μl of the extracted *Giardia* DNA template, 1× KOD-Plus buffer, 0.4 μM of each primer, 0.2 mM of each dNTP, 1 mM of $MgSO_4$, and 1 U of KOD-Plus DNA Polymerase (TOYOBO, Osaka, Japan), with 5% DMSO as an additive. The cycling parameters were as follows: initial denaturation at 94 °C for 30 s; followed by 30 cycles at 94 °C for 20 s, 63.4 °C for 30 s, 72 °C for 45 s; and a final extension at 72 °C for 3 min. The reaction mixture and cycle parameters for secondary PCR were the same as those used for the primary PCR except for the following 2 steps: (1) the initial denaturation step, which was performed for 30 s instead of 20 s and (2) the annealing step, which was performed at 62 °C instead of 63.4 °C. Electrophoresis and visualization of the amplified products and purification of the target fragments were performed following the same procedures as used for the 18S rRNA gene locus.

2.5. DNA sequence analysis

The purified PCR products containing the 18S rRNA gene locus were directly sequenced with appropriate primers in both directions on ABI Prism 310 Genetic Analyzer by using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, California, USA). The purified PCR products containing the GDH gene locus were also sequenced directly as described above. However, some of the PCR products, i.e., those containing the fragments of the GDH gene isolated

Table 2
GDH gene sequences used in this study.

Isolate name[a]	Accession number	Assemblage[b]	Place of origin	Reference
Ad-1	AY178735	A-I	Australia	[10,32]
Ad-2	L40510	A-II	Australia	[10,32]
BAH-12	AF069059	B-III	Australia	[10,32]
Ad-7	L40508	B-IV	Australia	[10,32]
Ad-136	U60982	C (Dog)	Australia	[33]
Ad-148	U60986	D (Dog)	Australia	[33]
P-15	U47632	E (Livestock)	Australia	[10]
Ad-23	AF069057	F (Cat)	Australia	[10]
Ad-157	AF069058	G (Rat)	Australia	[10]
GH-135	AB195224	B	Japan	[21]
Ad-156	AY178752	B2 (Marmoset)	Australia	Direct submission
Ad-158	AY178753	B2 (Marmoset)	Australia	Direct submission
gi-hum1	DQ840541	B	Poland	Direct submission
gi-cat1	DQ840542	B (Cat)	Poland	Direct submission
NLH25	AY826193	B	Netherlands	[12]
NLH35	AY826197	B	Netherlands	[12]
gd-ber4	DQ090535	B	Norway	[15]
PalH4-1	AB295649	B	Palestine	This study
PalH4-2	AB295650	B	Palestine	This study
PalH4-3	AB295651	B	Palestine	This study
PalH8-1	AB295652	B	Palestine	This study
PalH8-2	AB295653	B	Palestine	This study
PalH8-3	AB295654	B	Palestine	This study
–	AF069065	*Giardia ardeae*	–	[10]

[a] Isolate names and genotypes are derived from the DDBJ/GenBank/EMBL database and reference information.

[b] Information in parentheses indicates the source of those samples that were not obtained from human subjects.

from 3 samples (PalH4, PalH6, and PalH8) were subcloned into the EcoRV site of pBluescript II SK(+) (Stratagene, California, USA), and their full-length sequences were determined using T3 and T7 primers with appropriate internal sequencing primers. A subcloning strategy was adopted to determine the sequences from the above mentioned samples, because mixed-genotype infection profiles were observed in 2 samples (PalH4 and PalH8). The sample PalH6 was also analyzed by subcloning as a control sample of non-mixed infection. At least 2 independent PCR products were used in this process to confirm the sequencing results. DNA sequences were analyzed using the DNASIS-Mac v3.6 (Hitachi, Yokohama, Japan).

2.6. Sequence alignment and phylogenetic analysis

All reference sequences of the 18S rRNA and GDH genes of *G. intestinalis* used in this study were obtained from the DNA Data Bank of Japan (DDBJ) by using the blastn algorithm (http://blast.ddbj.nig.ac.jp/top-e.html). Alignments and phylogenetic analysis were performed by running the ClustalW v1.83 program on the DDBJ homepage (http://clustalw.ddbj.nig.ac.jp/top-e.html). An unrooted neighbor-joining (NJ) tree composed of the nucleotide sequences obtained in this study and 17 sequences of the GDH gene from various assemblages of *G. intestinalis* was reconstructed using TreeView v1.6.6 with *G. ardeae* as the outgroup. Branch lengths and bootstrap values (1000 replicates) were derived from the NJ analysis.

2.7 Nucleotide sequence accession numbers

The partial sequences of the GDH gene of *G. intestinalis* reported in the present study were submitted to the DDBJ/European Molecular Biology Laboratory (EMBL)/GenBank nucleotide sequence databases under accession numbers from AB295649–AB295654.

3. Results

3.1. Microscopic examination and patient background

Of the 69 fecal samples collected, 8 were found to be cyst positive on direct microscopic examination after purification by the sucrose centrifugal flotation method (Table 1). The patients comprised 6 males, 1 female, and 1 person of unknown sex, all in the age range of 1 to 36 years. All the patients complained of abdominal pain as the main symptom; however, no cases of watery diarrhea were observed. The cyst abundances in samples were revealed as a high cyst count (+++)

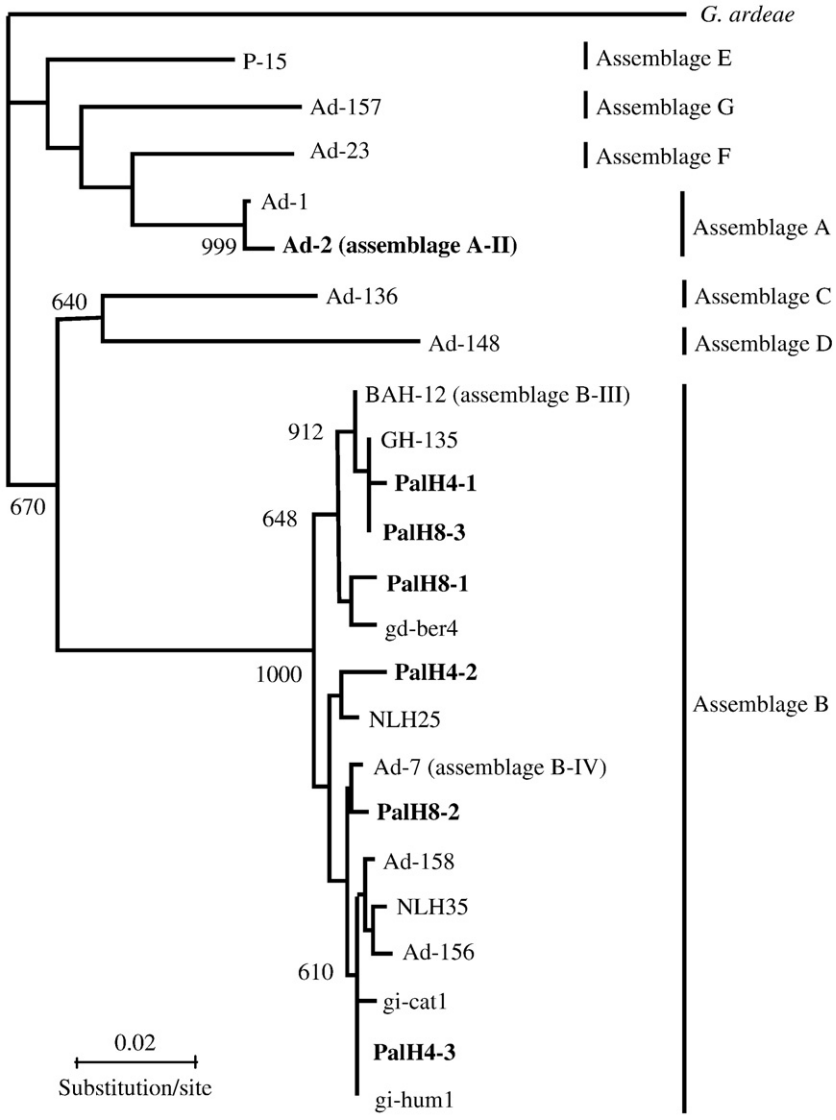

Fig. 1. NJ tree reconstructed with the GDH gene sequences obtained in this study and references of *G. intestinalis*. Six isolates from 2 mixed-infection samples and the assemblage A-II reference sequence (L40510), which was identical to the sequences of the subgenotypes from the PalH2, PalH5, PalH6 and PalH7 samples, are shown in bold. All reference sequences are shown by isolate names and their details are listed in Table 2. Numbers on nodes represent bootstrap values from 1000 replicates.

in the PalH4 and PalH8 samples, and a moderate (++) to low (+) cyst count in all the other samples.

3.2. 18S rRNA gene analysis

18S rRNA gene segments (375 bp) were successfully amplified from all the samples by PCR. All the 18S rRNA gene sequences obtained in this study showed 100% identity to the reference sequences. Two identical sequences from the PalH4 and PalH8 samples were homologous to a sequence of assemblage B (DDBJ/GenBank/EMBL accession number, AF199447) [25], and all the sequences from the other samples were identical to a sequence of assemblage A (AF199446) [25]. As observed in previous works [10,12], the results showed a comparatively low resolution due to the conserved nature of the 18S rRNA gene sequence, and could not be used to differentiate the sequences at the subgenotype level (data not shown). However, all the 18S rRNA gene analysis results were consistent with the GDH gene analysis results described below, and no discrepancy was observed in the determined genotypes (Table 1).

3.3. GDH gene analysis

GDH gene segments (393 bp) were successfully amplified by PCR and their full-length sequences were determined as described in "Materials and methods" from all the samples except PalH1 and PalH3. The GDH gene sequences from the PalH2, PalH5, PalH6, and PalH7 samples showed 100% homology to a sequence of assemblage A-II (L40510, Table 2). The PalH4 and PalH8 samples, whose sequences were determined using the subcloning technique as described in "Materials and methods," revealed complex mixed subgenotypes. Each sample contained at least 3 independent isolates (PalH4: PalH4-1, PalH4-2, and PalH4-3; PalH8: PalH8-1, PalH8-2, and PalH8-3), which were all clustered into assemblage B (Table 2, Fig. 1). There was no overlapping of subgenotype sequences among those samples. While the sequences of 6 clones of the control PalH6 sample showed complete identity to the direct sequencing result mentioned above, thus the possibility of PCR mutagenesis was denied in this methodology.

3.4. Analyses of the samples with mixed subgenotypes

Of the 34 clones isolated from the PalH4 sample, 44.1%, 20.6%, and 35.3% clones showed the PalH4-1, PalH4-2, and PalH4-3 subgenotypes, respectively, while of the 16 clones isolated from the PalH8 sample, 31.3%, 37.4%, and 31.3% clones showed the PalH8-1, PalH8-2, and PalH8-3 subgenotypes, respectively. A DDBJ/GenBank/EMBL database search revealed 2 homologues; the sequence of subgenotype PalH4-3 was identical to the unpublished sequence gi-hum1 (DQ840541) reported from Poland and that of subgenotype PalH8-3 was identical to GH-135 (AB195224) reported from Japan. The sequences of none of the other subgenotypes showed homology with any sequence in the database (Table 2, Fig. 1). Alignment of all the 6 confirmed nucleotide sequences of the clones revealed 12 single-nucleotide polymorphisms (SNPs) within the PalH4 subgenotypes and 10 SNPs within the PalH8 subgenotypes. As a result, a total of 15 SNPs were confirmed among the 6 subgenotypes (Table 3). Translation of all the GDH gene sequences of the subgenotypes revealed a conserved characteristic; the sequences of all the assemblage B subgenotypes were translated to a certain conserved amino acid sequence, since all the substitutions were silent and positioned at the third nucleotide of codons (Table 3).

Phylogenetically (Fig. 1), the GDH gene sequences of the subgenotypes of the PalH2, PalH5, PalH6, and PalH7 samples, all of which were completely homologous with a reference sequence of assemblage A-II (Ad-2), formed a cluster together with the reference sequence of assemblage A-I (Ad-1) with a high bootstrap value (99.9%). This cluster was clearly differentiated from an assemblage B cluster, which included all the mixed-subgenotype isolates and reference sequences from various organisms. Although the assemblage B cluster seemed widespread and highly divergent, the local bootstrap value (100%) was statistically significant and supported the formation.

4. Discussion

In the present study, we identified 2 cases of intra-assemblage mixed infections in a single fecal sample. The mixed infection may be because the patients had been repeatedly and cumulatively exposed to the pathogen due to poor sanitation in the study areas in Nablus, West bank, Palestine (Table 1). The only previous report on intestinal protozoan parasites in the area [26] showed the prevalence of intestinal parasites such as *Entamoeba histolytica* (22.9%), *G. intestinalis* (7.3%), and *Ascaris lumbricoides* (5.7%), thus supporting our speculation. In addition, the fact that a case of mixed infection is rarely reported in developed countries [12,17–19,27,28].

In contrast to a comparatively high prevalence of inter-assemblage mixed infections, which were detected using common methods such as restriction fragment length polymorphism [12,18,24,27,29] or PCR with assemblage-specific primer sets [8,12,24,30], the prevalence of intra-assemblage mixed infections, which were detected by their mixed nucleotide sequence profiles obtained by direct sequencing, has been rare [15,16,18]. Considering these facts together with the subcloning results obtained in this study, it appears that the incidence of intra-assemblage mixed infections is underestimated. Lalle et al. [18] stated that they reported only inter-assemblage mixed infections because of the technical difficulties encountered in the determination of intra-assemblage mixed infections. In other words, previous studies apparently failed to detect intra-assemblage mixed infections possibly due to the isolation process-related selection bias to in vitro culture of *G. intestinalis* and also due to the technical limitations of the above mentioned conventional methods. However, we analyzed the genomic DNA directly extracted from the fecal specimens and revealed the original DNA population structures in the samples by using a subcloning technique.

In addition, as shown by our results, 18S rRNA gene analysis could not detect the mixed-infection profiles, suggesting that highly

Table 3
Alignment of the GDH nucleotide sequences by using assemblage B subgenotypes isolated from the mixed-infection samples.

Reference and subgenotypes	Deduced amino acid and substituted nucleotide sequences														
	Asn	Leu	Leu	Pro	Gly	Phe	Cys	Thr	Gly	Ile	Tyr	Phe	Leu	Arg	Gly
	AA-87	CT-99	CT-147	CC-150	GG-156	TT-219	TG-222	AC-237	GG-258	AT-309	TA-330	TT-351	CT-363	AG-402	GG-417
AF069059	C	C	T	G	C	T	C	T	G	C	C	C	C	G	G
PalH4-1	C	C	T	G	C	T	C	T	G	T	C	C	T	G	G
PalH4-2	T	C	T	G	C	C	C	C	A	C	T	T	C	A	A
PalH4-3	C	T	C	G	C	C	C	C	G	C	T	C	C	A	G
PalH8-1	C	T	C	A	C	T	T	T	G	C	C	C	C	G	G
PalH8-2	C	T	T	G	T	C	C	C	G	C	T	C	C	A	G
PalH8-3	C	C	T	G	C	T	C	T	G	T	C	C	C	G	G

Substituted positions were determined by the alignment of 393-bp GDH gene fragments. Nucleotide position numbers were determined according to the reference assemblage B-III (AF069059), and substitutions at the third nucleotides of codons are shown by a hyphen "-". Genotypes of isolates from each mixed-infection sample consisted of 3 subgenotypes: PalH4 (PalH4-1, PalH4-2, and PalH4-3) and PalH8 (PalH8-1, PalH8-2, and PalH8-3).

polymorphic target gene loci such as the GDH gene locus are needed for precise evaluation of mixed infections. It is also suggested that the high levels of diversity observed within assemblage B could provide a suitable target for evaluating intra-assemblage mixed infections, while assemblage A does not seem to be suitable for the purpose because of its conservative features.

Similar to *Giardia* genotyping reports from other geographic areas, e.g., USA [17], UK [27], Netherlands [12,19], Italy [18,28], India [16,17], China [30], Mexico [31,32], and Australia [9,10,33], the samples from Palestine also showed only assemblage A and B and none of the remaining genotypes (C–G), which have mainly been reported in animals [4,10,16]. All the determined sequences of assemblage A samples from Palestine were completely identical to the sequence of reference assemblage A-II, which has been reported worldwide, e.g., in UK [27], Italy [28], India [16], Mexico [31,32], and Australia [10].

Although the phylogenetic analysis of the GDH gene sequences indicated a high degree of genetic polymorphism within the assemblage B cluster, alignment analysis of the polymorphisms within the GDH gene locus revealed that all nucleotide substitutions observed among the assemblage B subgenotypes were positioned at the third nucleotide of the codons and were transition mutations, i.e., they were purine–purine or pyrimidine–pyrimidine substitutions (Table 3). Due to the restricted substitutions, all the mutations on the nucleotide level were synonymous, resulting in no change in the deduced amino acid sequence. In other words, all the nucleotide substitutions converged on 1 amino acid sequence (Table 3). On the other hand, in assemblage A subgenotypes, all the reference sequences including those of assemblage A-II subgenotypes of the GDH gene appeared to converge on an another amino acid sequence (data not shown).

These findings reasonably suggest that genetic diversity in *G. intestinalis* could be analyzed at 2 independent levels: (1) inter-assemblage amino acid diversity, which appears completely conserved within each assemblage and may reflect unrevealed ancient divergence and (2) intra-assemblage nucleotide diversity, which may reflect an ongoing process of nucleotide mutations under the restriction of the former divergence. Although the driving force and maintenance mechanism of these 2 levels of diversity remain unclear, the concept could contribute to a better understanding of the evolution of *G. intestinalis*.

In conclusion, the concept of 2 levels of diversity could be useful for elucidating the evolutionary patterns and present population structure of *G. intestinalis*. Furthermore, the comparatively high prevalence of intra-assemblage mixed infections of *G. intestinalis* observed in this study indicates the common occurrence of repeated and cumulative infections by the pathogen, especially in endemic areas. Therefore, a subcloning approach targeting highly polymorphic gene loci is recommended to obtain precise and detailed molecular epidemiological data, which could directly contribute to a better understanding of the intraspecific diversity of this unique pathogen.

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