RESEARCH NOTE

IMPROVED SENSITIVITY OF PCR AMPLIFICATION OF GLUTAMATE DEHYDROGENASE GENE FOR DETECTION AND GENOTYPING OF *GIARDIA DUODENALIS* IN STOOL SPECIMEN

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Abstract. A modified set of primers was developed to increase the sensitivity of nested PCR amplification of glutamate dehydrogenase (*gdh*) gene to detect and genotype *Giardia duodenalis* cysts in stool specimens. This modified set of primers had a significantly higher sensitivity (82%) than that of a previously published PCR primer set (53%).

Key words: *Giardia duodenalis*, PCR, glutamate dehydrogenase gene, sensitivity

INTRODUCTION

Giardia duodenalis is an intestinal flagellate protozoan which infects a wide range of hosts, *eg*, humans, livestock and domestic animals (Thompson, 2000). Molecular studies reveal that *G. duodenalis* is a complex species with genotypic distinctions (Mayrhofer *et al*, 1995; Monis *et al*, 1996, 1999, 2003). Two major genetic assemblages, A and B, of *G. duodenalis* are recovered from humans (Mayrhofer *et al*, 1995; Monis *et al*, 1996, 2003). Assemblage A has two distinct clusters, AI and AII, while assemblage B consists of BIII and BIV (Monis *et al*, 1996).

Genotypic characterization of *G. duodenalis* has been shown to be a useful tool in epidemiological studies or outbreak investigations (Robertson *et al*, 2006; van der Giessen *et al*, 2006). PCR techniques for genotyping of *G. duodenalis* are based on polymorphic genes encoding 18S rRNA, glutamate dehydrogenase (*gdh*), elongation factor 1-alpha (ef1-α), triose phosphate isomerase (*tpi*), and β-giardin (Ey *et al*, 1997; Monis *et al*, 1998, 1999). PCR amplification and RFLP/sequence analysis of all of these genes with the exception of the 18S rRNA can differentiate subgenotypes of assemblage A (Traub *et al*, 2004). PCR methods for the detection of *gdh* can provide information on *G. duodenalis* A and B subgenotypes (Monis *et al*, 1996; Homan

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et al, 1998; Read *et al*, 2004). However, the sensitivity of PCR amplification of the *gdh* gene is rather limited, particularly when low numbers of cysts are present in fecal samples (Bertrand *et al*, 2005; Nantavisai *et al*, 2007).

A number of primer sets have been developed to amplify *gdh* by semi-nested PCR (Homan *et al*, 1998; Read *et al*, 2004). This study aimed to increase the sensitivity of nested PCR of *gdh* for the detection of *G. duodenalis* in fecal specimens using a modified set of PCR primers.

MATERIALS AND METHODS

Non-preserved fecal specimens of *G. duodenalis* were obtained from a survey of intestinal parasitic infections in an orphanage, schools and rural communities, which have been approved by the ethics committee of the Royal Thai Army Medical Department. Fecal specimens were examined for *G. duodenalis* cysts under light microscopy using wet preparation, and then *G. duodenalis* cysts were concentrated using a sodium nitrate flotation technique (O' Grady and Slocombe, 1980). In brief, stools were suspended in phosphate-buffered saline (PBS), filtered through gauze and approximately 2 g of stool were mixed thoroughly with saturated $NaNO_3$ and left for 20 minutes. Cysts at the top 1 ml of supernatant were collected from each sample, washed three times with PBS, resuspended in PBS and kept at -20ºC until used.

Giardia DNA was extracted from FTA disks (Whatman, Bioscience, USA) as previously described (Nantavisai *et al*, 2007). Fifteen μl of specimen were applied onto a 6 mm-diameter FTA disk which was allowed to air-dry overnight. The disk was cut into 4 pieces and each piece was used in the test. The FTA disk was washed twice with 200 μl of FTA purification buffer (Life technologies, Gaitherburg, MD) for 15 minutes, washed twice with 200 μl of TE^{-1} buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) for 5 minutes and dried overnight. The washed FTA disks were used as DNA templates in semi-nested PCR amplification. In addition, a QIAmp Stool Mini Kit (Qiagen, Germany) was used for *Giardia* DNA extraction of fecal specimens that gave negative PCR results with FTA disks.

To design primers, sequence information of *gdh* was obtained from the NCBI database: GenBank accession number L40509 (assemblage AI) (Monis *et al*, 1996); L40510 (assemblage AII) (Monis *et al*, 1996); AF069059 (assemblage BIII) (Monis *et al*, 1999); L40508, AY826191, AY826192, and AY826193 (assemblage BIV) (Monis *et al*, 1996; van der Giessen *et al*, 2006); U60984 (assemblage C) (Monis *et al*, 1998); U60986 (assemblage D) (Monis *et al*, 1998); U47632, AY826198, AY826199, and AY826200 (assemblage E) (Monis *et al*, 1998; van der Giessen *et al*, 2006). The sequences were aligned using ClustalW version 1.83 (Thompson *et al*, 1994). To amplify *gdh*, a primary external forward primer, GDH1a (5'ATCTTCGAGAAGGATGCT TGAG3') and external reverse primer, GDH5s (5'GGATACTTSTCCTTG AACTC3') were developed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm). Modified primers were designed to cover all cognate sequences and to enable amplification of isolates across all assemblages. The primer GDH1a was designed based on primer GDH1 (Homan *et al*, 1998) with one more adenine base inserted. This insertion was added according to the sequence variation at the priming position of primer GDH1 deposited in Genbank (AY826191, AY826192, AY826193, AY826198, AY826199, and AY826200). Using primer GDH1 together with primer GDH1a will cover all

Table 1
Sequences of PCR primers of the *gdh* gene used for the detection of *G. duodenalis* cysts in this study.

PCR	Directions	Primers	Fragment sizes (bp)	References
Primary	Forward	GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3')	2324	This study
	Forward	GDH1a (5'ATC TTC GAG AAG GAT GCT TGA G3')		
	Reverse	GDH5s (5'GGA TAC TTS TCC TTG AAC TC3')		
Secondary	Forward	GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3')	461	Read *et al*, 2004
	Reverse	GDHiR (5'GTT RTC CTT GCA CAT CTC C3')		
Primary	Forward	GDH1 (5'ATC TTC GAG AGG ATG CTT GAG3')	770	Homan *et al*, 1998
	Reverse	GDH4 (5'AGT ACG CGA CGC TGG GAT ACT3')		
Secondary	Forward	GDHeF (5'TAC ACG TYA AYC GYG GYT TCC GT3')	461	Read *et al*, 2004
	Reverse	GDHiR (5'GTT RTC CTT GCA CAT CTC C3')		

Y= C or T; R = A or G

variations existing at these positions. The GDH5s primer contained one degenerate base, S, where S = G or C. In the primary PCR amplification, primers GDH1, GDH1a and GDH5s span *gdh* domain from base +138 to base +2461 which leads to the production of 2324 bp fragment. Compared to primers described by Homan *et al* (1998), the forward primer GDH1 (5'ATCTT CGAGAGGATGCTTGAG3') and reverse primer GDH4 (5'AGTACGCGACGC TGGGATACT3') span *gdh* domain from base +138 to base +907 and produced 770 bp fragment. By using the same set of primers in the secondary PCR, a 461 bp of *gdh* was amplified using GDHeF (5'TACA CGTYAAYCGYGGYTTCCGT3') (Y = C or T) and GDHiR (5'GTTRTCCTTGCA CATCTCC3') (R = A or G) (Table 1). The restriction sizes of the amplicons digested with *Nla*IV and *Rsa*I were predicted using program ApE-A plasmid Editor version 1.11.

The first-round PCR amplification was performed using mixtures of 2 U of *Taq* polymerase with 1X PCR buffer, 2.5 mM $MgCl_2$, 250 mM of each dNTP, 25 pmol of each primer, and 2 pieces of FTA disks (or 1-2 μl of the extracted DNA) in a total volume of 50 μl. The thermal cycling conditions were as follows: 94ºC for 7 minutes and then 35 cycles of 94ºC for 1 minute, 55ºC for 1 minute, and 72ºC for 1 minute, with a final cycle of 72ºC for 7 minutes. The second-round PCR was performed using mixtures of 2U of *Taq* polymerase with 1X PCR buffer, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 25 pmol of each

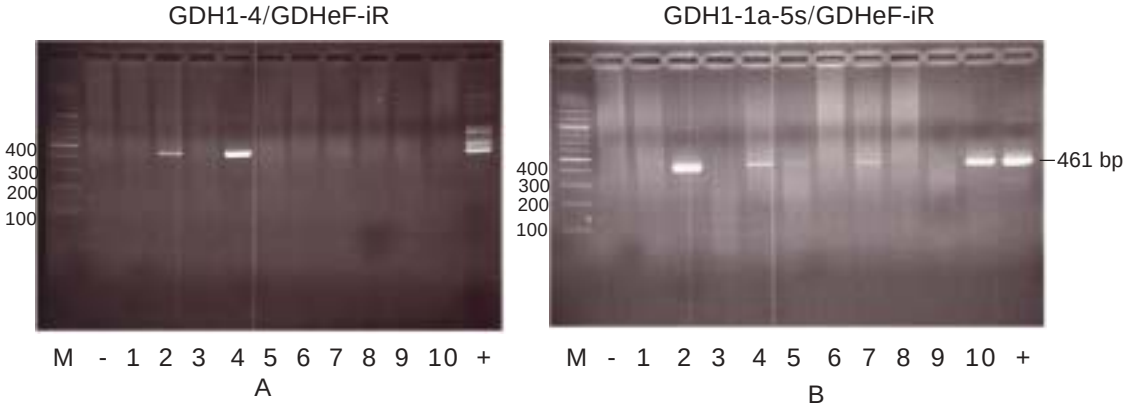

Fig 1–Nested PCR amplification of *G. duodenalis gdh* using two primer sets. Lane M, 100-bp molecular markers; lane -, negative control; lanes 1 to 10, stool specimens; lane +, positive control.

primer, and 1 μl of the primary PCR product in a total volume of 50 μl. The thermal cycling was initiated with 1 cycle of 94ºC for 2 minutes, 56ºC for 1 minute, and 72ºC for 2 minutes, followed by 55 cycles of 94ºC for 30 seconds, 56ºC for 20 seconds, 72ºC for 45 seconds, and final extension at 72ºC for 7 minutes. The PCR products were analyzed by 2% agarose gel-electrophoresis, stained with ethidium bromide and then visualized on a UV transilluminator. DNA extracts of *G. duodenalis* assemblage AI from an axenic culture was used as positive control throughout the study.

RESULTS

A total of 66 *G. duodenalis*-positive specimens were analyzed by nested PCR using amplification of the *gdh* gene. Using GDH1/GDH4 primers and secondary primers of GDHeF/GDHiR, 35 (53%, 95% CI 40.3-65.4) were successfully amplified, whereas 54 (82%, 95% CI 70.4-90.2) were PCR positive using the modified primary primers (GDH1/GDH1a/GDH5s) and secondary primers (GDHeF/GDHiR). The sensitivity of modified primer set (GDH1/GDH1a/GDH5s) was significantly higher than that of GDH1/GDH4 primers (chi-square, $p < 0.05$). Use of GDHeF/GDHiR as secondary primers showed satisfactory results (Fig 1). Of 20 fresh stool samples, use of GDH1/GDH1a/GDH5s and GDHeF/GDHiR primer set showed sensitivity of 95% (19 of 20), while GDH1/GDH4 and GDHeF/GDHiR primer set gave 60% sensitivity (12 of 20). Beneficially, the diagnostic genotyping profiles of Read *et al* (2004) still could be used to analyze RFLP patterns. Of 57 samples, RFLP analysis classified 12 samples (21%) into assemblage AI, 17 samples (30%) assemblage AII, 2 samples (4%) assemblage BIII and 26 samples (46%) assemblage BIV.

DISCUSSION

Nucleotide polymorphisms of *gdh* provides a rapid means to identify all subgenotypes of *G. duodenalis*. This study provided a modified set of primers for nested PCR amplification of *gdh* for the detection and genotyping of *G. duodenalis* in human fecal specimens. False negative PCR reaction could have ocurred due to a long storage of stool specimens. In our experience, working on fresh stool specimens and immediately collecting *Giardia* cysts by flotation method gave better

results of the sensitivity than those stored at 4ºC for a few months.

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