

## Evaluation of the Sensitivities of DNA Extraction and PCR Methods for Detection of *Giardia duodenalis* in Stool Specimens<sup>∇</sup>

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**Sensitivities of DNA extraction methods and PCR methods for *Giardia duodenalis* were evaluated. A combination of the most sensitive methods, i.e., FTA filter paper and a PCR protocol using RH11/RH4 and GiarF/GiarR primers, showed no significant differences compared to immunofluorescence assay in terms of their sensitivities and specificities.**

*Giardia duodenalis* is an intestinal flagellate that infects humans and other mammals, including pets and livestock, throughout the world. Approximately  $2.8 \times 10^8$  people are infected by this organism each year (5). Light microscopy or immunofluorescence assay has been used to identify *G. duodenalis* in most laboratories. However, these techniques might not be sensitive enough to detect low numbers of excreted cysts (4). In addition, these methods cannot be used to differentiate genotypes of *G. duodenalis*. Recently, a few PCR-based techniques have been developed for detection and genotypic characterization of *G. duodenalis* (2, 3, 6, 9, 11). However, PCR techniques using stool specimens could be insensitive because of PCR inhibitors and the difficulty of cyst disruption. To raise the sensitivity of PCR, an effective DNA extraction method is needed. Commercial DNA extraction kits such as the QIAamp stool minikit (QIAGEN, Hilden, Germany) and FTA filter paper (Whatman Bioscience, Cambridge, United Kingdom) have been used for isolation of *Giardia* DNA (8, 12). However, these DNA extraction methods have never been compared. To determine the efficiencies of the three DNA extraction methods, i.e., FTA filter paper (Whatman Bioscience, United Kingdom), the QIAamp stool minikit (QIAGEN, Germany), and the conventional phenol-chloroform method, a known number of *G. duodenalis* cysts was used. Cysts of *G. duodenalis* were concentrated by saturated sodium nitrate flotation from a positive specimen collected from an asymptomatic member of the army during an annual health examination. The sample was washed three times with phosphate-buffered saline, followed by cyst counting using a hemocytometer. The sample was then 1:5 serially diluted to obtain 16,842, 3,368, and 674 cysts/ml. In addition, a 1:2 serial dilution of sample containing 674 cysts/ml was performed (to give 337 and 168 cysts/ml). These solutions containing different number of cysts were used for three DNA extraction methods. Since 200  $\mu$ l of each dilution was used for DNA isolation by the QIAamp stool minikit and conventional

phenol-chloroform methods and only 10  $\mu$ l of each dilution was used as DNA template in PCR amplification, the numbers of cysts per PCR were 168, 34, 7, 3, and 2, respectively. In the FTA method, the amount of specimen placed on the FTA disk was limited to 15  $\mu$ l, and only one-fourth of the FTA disk was used for each test. Thus, the numbers of cysts per PCR were equivalent to 63, 13, 3, 1.3, 0.6.

For DNA extraction with the FTA filter paper, the filter disk was allowed to air dry overnight after the application of specimens. The one-fourth piece of FTA disk was washed twice with 200  $\mu$ l of FTA purification reagent (Life Technologies, Gaithersburg, MD) for 15 min and then washed twice again with 200  $\mu$ l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) for 15 min and air dried overnight. The washed paper was then used directly as the DNA template in PCR amplification. For the QIAamp stool minikit (QIAGEN, Germany), 200  $\mu$ l of each diluted sample was used for DNA extraction, following the manufacturer's instructions. The extracted DNA of each sample was kept frozen at  $-20^\circ\text{C}$  until used. The phenol-chloroform extraction was performed as described by Hopkins et al. (3). The most efficient extraction method was defined as the method that could extract DNA from the lowest cyst numbers and that gave a positive band of *G. duodenalis* using the RH11/RH4-GiarF/GiarR primer set with the PCR conditions described by Hopkins et al. (3).

The comparison showed that FTA filter paper was the most efficient DNA extraction method; it could detect as few as 168 cysts/ml, while both the QIAamp stool minikit and phenol-chloroform extraction method could detect 674 cysts/ml stool dilution. In addition to its high sensitivity, the FTA filter paper assay was simple to use and can be applied with a large number of samples at one time. The samples are also easy to handle and transport for further analysis. The major disadvantage of using this procedure may be that some parts of the disk may contain more DNA template than other parts. This can affect the result of PCR amplification. To handle this problem, at least two PCR amplifications per disk of FTA filter paper are recommended.

PCR techniques for detection of *G. duodenalis* are based on the polymorphic nature of *G. duodenalis* DNA sequences of

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TABLE 1. Comparison of the sensitivities of five PCR methods for the detection of *G. duodenalis* trophozoite DNA and cysts in stool specimens

Primers	Target gene locus	Expected amplicon size (bp)	Reference	PCR result <sup>a</sup> for:									
				Trophozoite DNA concn (pg/μl) of:						Cyst concn (cysts/ml) of:			
				100,000	10,000	1,000	100	10	1	3,368	674	337	168
RH11/RH4 and GiarF/GiarR	SSU rRNA	130	3	+	+	+	+	+	-	+	+	+	+
G7/G759 and forward/reverse	β-Giardin	511	2	+	+	+	+	-	-	-	-	-	-
GDHeF/GDHiR and GDHiF	Glutamate dehydrogenase	432	9	+	+	+	-	-	-	+	+	+	-
AL3543/AL3546 and AL3544/AL3545	Triosephosphate isomerase	530	11	+	+	+	-	-	-	+	-	-	-
EF1AR/GLONGF and RTef1-αF/RTef1-αR	Elongation factor 1-alpha	191	6	+	+	-	-	-	-	-	-	-	-

<sup>a</sup> All PCR amplifications were done in triplicate. +, at least one positive result out of three attempts.

the small-subunit (SSU) rRNA, glutamate dehydrogenase (GDH), elongation factor1-alpha (ef1-α), triosephosphate isomerase, and β-giardin genes (2, 3, 6, 9, 11). However, these PCR methods have never been compared. We determined the sensitivities of PCR primers with *G. duodenalis* trophozoite DNA as a template. Genomic DNA was extracted from assemblage B of *G. duodenalis* axenic trophozoites provided by the Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, using the QIAamp stool minikit (QIAGEN, Germany). After DNA extraction, the concentration of the DNA sample was determined by UV absorption at 260 nm. *G. duodenalis* trophozoite DNA was then serially diluted 1:10 (to 100,000, 10,000, 1,000, 100, 10, and 1 pg/μl, respectively) and used for PCR amplification with different primer sets, i.e., RH11/RH4 and GiarF/GiarR (3), GDHeF/GDHiR and GDHiF (9), AL3543/AL3546 and AL3544/AL3545 (11), G7/G759 and forward/reverse (2), and EF1AR/GLONGF and RTef1-αF/RTef1-αR (6). Genomic DNA and the primer sets were used with previously described PCR conditions (2, 3, 6, 9, 11). The most sensitive primer pair was defined as the one that could amplify DNA of *G. duodenalis* at the lowest trophozoite DNA concentration. The results showed that RH11/RH4 and GiarF/GiarR primer set, amplifying the SSU rRNA gene, was the most sensitive primer set and could detect as little as 10 pg of DNA/PCR mixture (Table 1).

We then determined the sensitivities of these primers for the detection of *G. duodenalis* (assemblage B) cyst DNA. DNA was extracted from stool specimens with four different concentrations of *G. duodenalis* cysts (i.e., 3,368, 674, 337, and 168 cysts/ml) by the FTA filter paper method. PCR amplifications were performed as described above. The most sensitive primer pair was defined as the one that could amplify DNA of *G. duodenalis* at the lowest numbers of cysts. The results demonstrated that the RH11/RH4 and GiarF/GiarR primer set was also the most sensitive primer set, which could detect as low as 168 cysts/ml (Table 1). The sensitivity of this primer set may be due to high copy numbers of the SSU rRNA gene in the organism. The presence of approximately 60 to 130 copies of rRNA gene per nucleus of *G. duodenalis*, arranged in tandem repeats, has been reported (1, 10). Moreover, the SSU rRNA sequence is more conserved than the other regions that were used as templates, which could be the explanation for why primers amplifying the SSU rRNA gene worked better.

The development of immunofluorescence assays has gener-

ally improved the sensitivity of detecting and quantitating fecal *Giardia* cysts and may allow accurate determination of prevalence rates and cyst excretion intensities compared to conventional microscopy (7). Although both immunofluorescence assay and PCR have been widely used, comparisons of sensitivities and specificities between these two procedures using human stool specimens have not been done. However, a study using calf stool specimens indicated that PCR was more sensitive than immunofluorescence assay (13).

Based on the above results, the most sensitive DNA extraction method (i.e., FTA filter paper) together with the most sensitive PCR method (i.e., that with the RH11/RH4 and GiarF/GiarR primer set) was compared to immunofluorescence assay in terms of sensitivities and specificities. Blinded evaluation of both techniques was performed using 70 stool samples (37 positive and 33 negative samples) which were collected from asymptomatic schoolchildren during a survey of parasitic infections in a primary school in Chachengsao Province, Thailand, in February 2006. *Giardia* cysts were microscopically identified using saturated sodium nitrate flotation. Stool specimens were stained with immunofluorescence antibody (Cellabs, United Kingdom) following the manufacturer's instructions and examined by fluorescence microscopy. Sensitivities and specificities were calculated using two-by-two tables and Epi Info version 6.04 software. A chi-square test was used to determine the significance between two proportions for sensitivities and specificities of the two diagnostic methods.

Using saturated sodium nitrate flotation as the gold standard, the sensitivities of the PCR method and the immunofluorescence assay were 97.3% (95% confidence interval [95% CI], 87.9 to 99.9%) and 91.9% (95% CI, 79.5 to 97.9%), respectively. The specificity of both PCR and immunofluorescence assay was 100% (95% CI, 91.3 to 100%). The difference between the sensitivities of both methods was 5.4% (95% CI, 0.9 to 16.7%). There were no significant difference between the sensitivities and specificities of the PCR and immunofluorescence assay ( $P = 0.61$ ).

In conclusion, several PCR methods for the detection of *G. duodenalis* have been published; however, there was no standardized method. The present study has identified a highly sensitive PCR method for the detection of *G. duodenalis* by using FTA filter paper for DNA extraction together with the RH11/RH4-GiarF/GiarR primer set amplifying the SSU rRNA gene.

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