

## Multiplex Real-Time PCR Assay Using Scorpion Probes and DNA Capture for Genotype-Specific Detection of *Giardia lamblia* on Fecal Samples

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Two major genotypic assemblages of *Giardia lamblia* infect humans; the epidemiologic significance of this phenomenon is poorly understood. We developed a single-vessel multiplex real-time PCR (qPCR) assay that genotypes *Giardia* infections into assemblages A and/or B directly from fecal samples. The assay utilized Scorpion probes that combined genotype-specific primers and probes for the 18S rRNA gene into the same molecule. The protocol was capable of detecting as few as 20 trophozoites per PCR on fecal DNA isolated using a commercial method or 1.25 trophozoites per PCR on fecal DNA isolated using a *G. lamblia*-specific oligonucleotide capture technique. The assay was specific for fecal specimens, with no amplification of the discordant genotype with the opposite Scorpion probe. When 97 clinical specimens from Bangladesh were used, the multiplex PCR assay detected 95% (21 of 22) of *Giardia* microscopy-positive specimens and 18% (13 of 74) of microscopy-negative specimens. Microscopy-negative and qPCR-positive specimens had higher average cycle threshold values than microscopy-positive and qPCR-positive specimens, suggesting that they represented true low-burden infections. Most (32 of 35) infections were assemblage B infections. This single-reaction multiplex qPCR assay distinguishes assemblage A *Giardia* infections from assemblage B infections directly on fecal samples and may aid epidemiologic investigation.

Members of the genus *Giardia* are the most commonly diagnosed intestinal parasites in the United States and cause approximately 200 million clinical infections per year worldwide (7). The genus can be distinguished on the basis of morphology, ultrastructural features, or 18S rRNA sequence into at least six species, *G. lamblia* (synonymous with *G. duodenalis* or *G. intestinalis*), *G. agilis*, *G. muris*, *G. ardeae*, *G. psittaci*, and *G. microti* (17). Isolates of *G. lamblia* have been further subgrouped by alloenzyme or sequence analysis of the glutamate dehydrogenase, triose phosphate isomerase, elongation factor 1 $\alpha$ , 18S rRNA, and other genes. Depending on the assay, *G. lamblia* subgroup nomenclature has included Nash groups 1 to 3 (19), genotype “Poland” versus “Belgium” (12), and assemblages A and B with subgroups A-I, A-II, B-III, and B-IV (16). Despite the complicated terminology, phylogenetic sequence analysis of the independent genetic loci has provided essentially concordant results showing that there are two major *G. lamblia* groups which cause human infection (14, 17). Use of the assemblage A and B nomenclature for these two main groups has been proposed, and we will adopt it hereafter for convenience.

While the genotypic separation of *G. lamblia* assemblages is

relatively well established, the clinical or epidemiologic significance of infection with assemblage A versus B is poorly understood. One report from Mexico (6) indicated that 11 isolates were assemblage A type, while another from Canada indicated that 12 of 15 clinical specimens were assemblage B type (9). Similarly small studies from India and Australia resulted in reports that assemblage A was associated with symptomatic infection relative to assemblage B (20, 22). In contrast, a correlation between assemblage B and persistent diarrheal symptoms was observed in The Netherlands (11).

More complete data on the epidemiology of infection with individual *Giardia* genotypes may enhance the clinical significance of detection and aid outbreak investigation. Existing diagnostic methods for use on human feces such as microscopy and enzyme-linked immunosorbent assay (ELISA) do not discriminate between the two assemblages. Genotype-specific PCR or PCR-restriction fragment length polymorphism has been performed by several investigators (3, 5, 10, 18) but is a time-consuming two-step procedure subject to contamination. Several real-time PCR (qPCR) assays for *Giardia* have subsequently been published (4, 8, 26, 27), including three that are capable of genotyping (2, 9, 13); however, most require multiple reactions, and details on assay sensitivity are unclear. Lastly, an assay using multiplex PCR and microarray hybridization has been published; however, that assay used only cultured trophozoites (28). We therefore sought to develop a qPCR assay utilizing self-probing amplicon primers (30) that would distinguish assemblages A and B in a single reaction and

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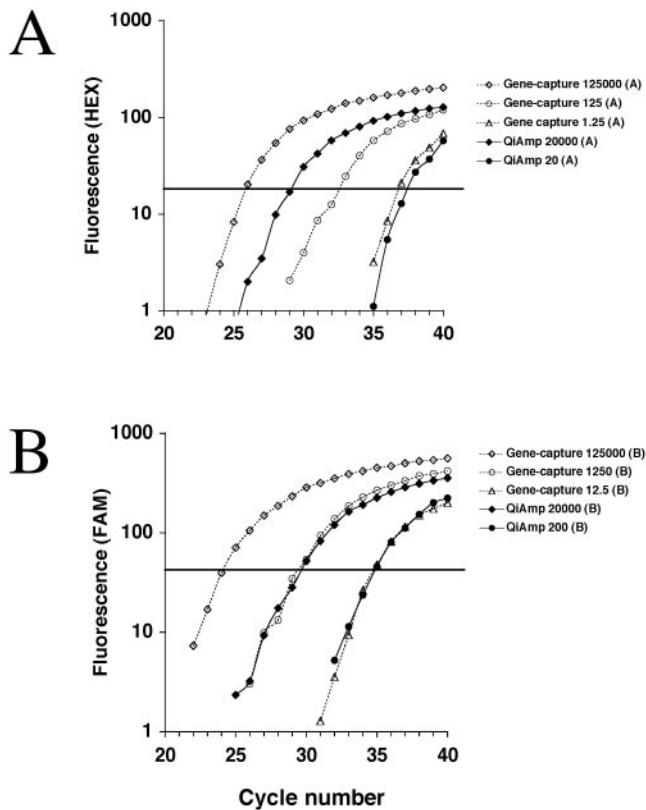


FIG. 2. Sensitivity of qPCR assay for *G. lamblia* assemblages A and B. Dilutions of assemblage A or B trophozoites were spiked into parasite-free stool, DNA was extracted using either the QIAamp (solid lines) or oligonucleotide-capture (hatched lines) method, and singleplex qPCR amplification was performed using Scorpion probes specific for assemblage A (A) or assemblage B (B). Legend values indicate numbers of spiked trophozoites per PCR. No fluorescence was observed with stool samples spiked with up to 20,000 trophozoites of the opposing assemblage/reaction or with unspiked stool (data not shown). Horizontal line indicates fluorescence threshold.

Amplification was performed on an iCycler (Bio-Rad, Hercules, Calif.) under the following cycling conditions: 95°C for 15 min and then 40 cycles of 94°C for 20 s and 60°C for 30 s. Amplification was confirmed in all reactions by gel electrophoresis; therefore, a 5-min 72°C incubation was added at the end of the cycling protocol.

**Statistics.** Values for the average threshold of qPCR amplification (cycle threshold [ $C_T$ ]) were compared between microscopy-positive and microscopy-negative groups by the Mann-Whitney test. The nonparametric Spearman correlation was used to quantitate the degree of association between ELISA optical density and  $C_T$  on ELISA-positive and qPCR-positive specimens.

## RESULTS

**Sensitivity of singleplex qPCR.** The sensitivity of the assay using the QIAamp extraction method to detect *G. lamblia* DNA in stool was  $\leq 20$  trophozoites per reaction for assemblage A ( $C_T = 37.4$ ) and  $\leq 200$  trophozoites per reaction for assemblage B ( $C_T = 35.0$ ) (Fig. 2). Sensitivity was increased with the addition of BSA and DMSO and was not enhanced by dilution of the DNA samples (data not shown), suggesting that the DNA yield was limiting as opposed to PCR inhibition. We therefore utilized a DNA extraction method involving sequence-specific gene capture of a downstream region of the

18S rRNA gene conserved between assemblages (Fig. 1A). DNA obtained by this method amplified at least 16-fold more efficiently than the QIAamp extraction procedure (equal or greater fluorescence at 1.25 versus 20 trophozoites/reaction for assemblage A and 12.5 versus 200 trophozoites/reaction for assemblage B; Fig. 1).

**Specificity of singleplex qPCR.** The specificity of the assemblage A- and B-specific Scorpion probes was tested on genomic DNA from *G. lamblia* assemblages A and B as well as on DNA isolated from stool samples spiked with up to 20,000 assemblage A or B trophozoites per reaction. No amplification was detected by qPCR or gel electrophoresis on discordantly spiked stool samples, regardless of parasite concentration or the method of DNA extraction (data not shown). Amplification of the discordant *G. lamblia* assemblage was only observed when pure genomic DNA ( $9.9 \times 10^5$  trophozoites/reaction) was used; such amplification was extremely inefficient, yielding a  $C_T$  equivalent to that obtained using 30 trophozoites of the concordant assemblage/reaction (data not shown). No amplification was observed with either Scorpion probe upon using genomic DNA of *E. histolytica*, *E. dispar*, *E. moshkovskii*, or *Cryptosporidium parvum* (data not shown).

**Multiplex PCR.** The two singleplex assays were combined to create a single-tube multiplex assay for use with human stool samples (Fig. 3). The test was specific, with no FAM fluorescence observed with assemblage A-spiked fecal samples (Fig. 3A) and no HEX fluorescence observed using B-spiked fecal samples (Fig. 3B). Sensitivity remained at the level of the singleplex assays after optimization for magnesium, dNTP, and *Taq* polymerase concentrations per procedures described above in Materials and Methods, and  $C_T$  levels were not diminished in coinfecting versus mono-infected specimens (Fig. 3C versus 3A or 3B).

**Comparison of multiplex PCR with microscopy for human clinical specimens.** Samples of 97 human stool specimens from diarrheal patients at the International Centre for Diarrheal Diseases, Bangladesh, were tested by saline wet-mount microscopy and by the qPCR assay using DNA extracted by the QIAamp method (Table 1). For confirmation, three of the PCR products (two from assemblage B and one from assemblage A) were sequenced and revealed complete identity to the published sequence (Fig. 1). The multiplex qPCR assay demonstrated 85% (83 of 97 samples) agreement with microscopy. The one microscopy-positive and qPCR-negative specimen was unavailable for additional testing; however, the microscopy-negative and qPCR-positive specimens were retested by ELISA, and 69% (9 of 13) were ELISA positive. Additionally, the microscopy-negative and qPCR-positive specimens had delayed  $C_T$  values versus the microscopy-positive and qPCR-positive specimens ( $35.3 \pm 4.5$  versus  $33.0 \pm 3.6$ ;  $P = 0.04$ ), further suggesting that the microscopy-negative and qPCR-positive specimens represented low-burden true infections. Finally, all available qPCR-positive specimens were retested by ELISA; 88% (30 of 34) were ELISA positive, and qPCR  $C_T$  values exhibited the expected inverse correlation with ELISA optical density values (correlation coefficient =  $-0.44$ ;  $P = 0.01$  by Spearman correlation test [ $n = 30$ ]). Accordingly, with qPCR as the “gold standard,” microscopic examination exhibited 98% specificity and only 62% sensitivity.

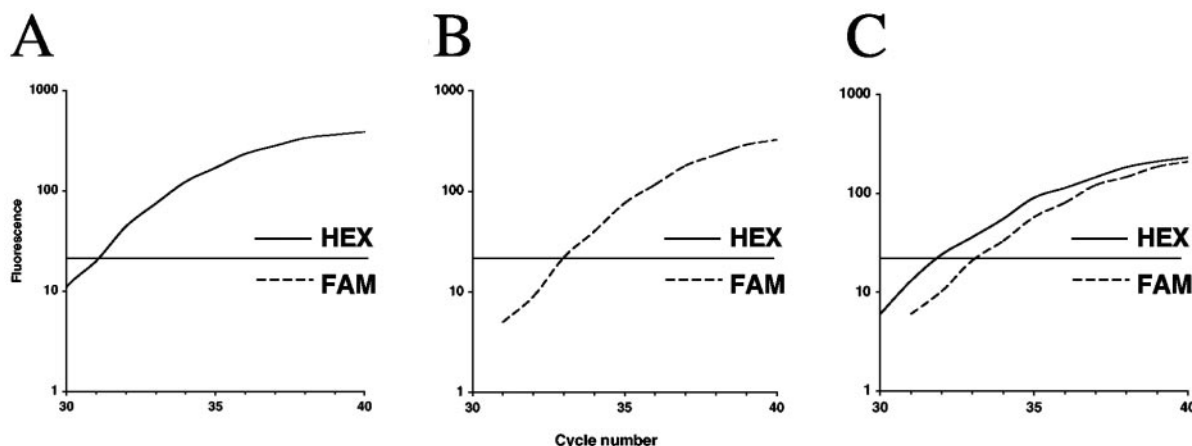


FIG. 3. Genotype-specific multiplex qPCR amplification for *G. lamblia* assemblages A and B. Parasite-free stool was spiked with 2,000 assemblage A trophozoites/reaction (A), 2,000 assemblage B trophozoites/reaction (B), or both (C). Multiplex qPCR was performed using the HEX-labeled assemblage A-specific Scorpion probe and the FAM-labeled assemblage B-specific Scorpion probe. No FAM fluorescence was detectable in the reaction represented by panel A, and no HEX fluorescence was detectable in the reaction represented by panel B.

DISCUSSION

This work details the development of a novel real-time PCR assay that genotypes *Giardia* infections from fecal specimens in a single closed-tube reaction. The test is specific and sensitive to well below the estimated parasite excretion rate in humans of 150 to 20,000 cysts/gram of stool (21). The assay can test 96 samples in 2 h and thus provides the capacity to characterize the *Giardia* genotype for large numbers of asymptomatic and symptomatic individuals from various geographic locations. Such epidemiologic data are presently limited but may ultimately enhance the ability to prognosticate an individual's course of infection and investigate source outbreaks.

We utilized Scorpion probes (30) instead of hydrolysis probes, hybridization probes, molecular beacons, or SYBR Green I. Although SYBR Green I was used for assay development (data not shown), we felt that the nonspecific fluorescence of an intercalating dye would be unacceptable given the complexity of fecally extracted DNA and our desire to multiplex. A published hydrolysis probe-based assay (13) was tried during early development; however, this TaqMan probe did not allow discrimination of genotypes and required a longer amplicon, which decreased amplification sensitivity (data not shown). Furthermore, in practice we found that genotype-specific design of TaqMan probes, hybridization probes, or molecular beacons was complicated by the high G-C content (~75%) of the *Giardia* 18S rRNA gene, its frequent runs of guanines, and the few nucleotide polymorphisms between as-

semblages. Moreover, these bi- or trimolecular reactions increase the possibility of primer-probe dimerization or nonspecific binding to DNA template and impose temperature constraints on the kinetics of hybridization, all of which would be magnified upon multiplexing.

Scorpion probes are unimolecular and yet can maintain a dual layer of specificity when both primer and probe are genotype specific. The primers we chose were specific for assemblage A and B isolates, including subgroups A-I and -II and B-III and -IV, according to available sequence. The primer segment of ScA contained a 3-bp mismatch to assemblage B DNA, and the probe portion took advantage of an additional 2-bp assemblage A-specific change. This dual layer of specificity ensured that falsely primed products would not lead to fluorescence detection. Sufficient specificity and sensitivity was achieved with an ScB Scorpion probe, with only a 3-bp mismatch at the primer 3' end, so a B-specific sequence was not required in the probe region. An additional layer of specificity to the PCR was added by utilization of a sequence-specific oligonucleotide to capture our DNA of interest. This DNA-capture technique (23) increased sensitivity at least 16-fold versus the results seen with the widely used commercial QIAamp stool kit, presumably due to increased target DNA yield. We used a proprietary lysis buffer for this method; however, others have performed the same technique with conventional reagents (15). On the same note, the multiplex Scorpion PCR can be rapidly cooled to 30°C for endpoint analysis on a fluorescent plate reader, obviating the expense of a real-time PCR machine. These are important concerns, as the assay should be as inexpensive as possible for the regions of the world where *Giardia* is highly endemic.

The test performed favorably with human diarrheal specimens and detected 60% more infections than microscopy alone. While there is no perfect gold standard test for enteric infections, we suspect that these microscopy-negative and qPCR-positive specimens were true infections given their high rate of ELISA positivity and relatively delayed  $C_T$ . The specimens were from a cohort of children with diarrhea in Mirpur,

TABLE 1. Comparison of multiplex qPCR and microscopy for diagnosis of *Giardia* infection

Microscopy result	No. of positive qPCR results ( $C_T \pm SD$ )			No. of negative qPCR results
	Assemblage A	Assemblage B	Total	
Positive	2	20	22 (33.0 $\pm$ 3.6) <sup>a</sup>	1
Negative	1	12	13 (35.3 $\pm$ 4.5)	61

<sup>a</sup>  $P = 0.04$ .

Bangladesh, and most (91%) were assemblage B infections. This majority is similar to the 70 to 80% rates of assemblage B infection observed in Australia (22) and Canada (9) but contrasts with reports from Mexico (6), India (20), and China (31), which have indicated equal or higher assemblage A infection rates. It is important to emphasize that the reported studies were small and uncontrolled and that some used cultivated trophozoites as opposed to direct fecal testing. As such, we will await larger studies, and this is the rationale for developing this multiplex assay.

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