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Real-Time Polymerase Chain Reaction for Detection of *Strongyloides stercoralis* in Stool

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Abstract. The use of real-time polymerase chain reaction (PCR) for detection of *Strongyloides stercoralis* in stool has recently been described. We compared five DNA extraction methods by using normal human stool spiked with *Strongyloides ratti* and tested by using a real-time PCR. The PowerSoil kit was found to be the best technique in terms of sensitivity and ease of use. The PCR detected DNA extracted from one spiked *S. ratti* larva diluted 10^{-2}. The PowerSoil kit was then used to extract DNA from 160 human survey samples. All culture positive specimens with a high and moderate larval load were identified by real-time PCR, but only 15% of specimens with low larval load were positive. Specificity was greater than 99%. The combination of the PowerSoil kit and real-time PCR reliably detected high to moderate larval numbers of *S. stercoralis* in stools but was less sensitive when the larval load was low.

INTRODUCTION

Definitive diagnosis of *Strongyloides stercoralis* relies on the detection of larvae. However, low and irregular larval output, especially in chronic infections, can hamper detection in stool and lead to an underestimation of infection rate.[1] Agar Plate Culture (APC) and Harada-Mori culture techniques are commonly used methods to detect live larvae in stool. The diagnosis of *S. stercoralis* in the stool is improved by multiple collections.[2–5] Serologic analysis is also used to diagnose strongyloidiasis. However, specificity may be decreased because of cross-reactivity with other helminths.[6–8] Improvements to serological methods such as isotype detection and enzyme-linked immunosorbent assay using recombinant antigen are under evaluation.[7,9]

Molecular methods are rapidly replacing the traditional culture methods. Real-time polymerase chain reaction (PCR) is reported to be specific and sensitive for the diagnosis of strongyloidiasis.[10–12] However, false-negative PCR results may occur because of the presence of inhibitory substances, and inhibition of PCR for viruses in human stool has been reported to occur in 0–44% of samples.[13–16] Thus, the ultimate sensitivity of a PCR depends on the efficient lysis of larva within the stool sample and the purification of target DNA free of inhibitors, which can interfere with the PCR.[17]

In this study, we compared different DNA extraction techniques and used the best of these techniques to evaluate the sensitivity and specificity of a real-time PCR. The efficiency of the chosen extraction method and the real-time PCR were then tested with 160 stool samples previously cultured for *S. stercoralis*.

MATERIALS AND METHODS

Spiking of human stool with *Strongyloides ratti*. First-stage larvae in stool were collected from laboratory rats after cutaneous exposure with 15,000 *S. ratti* third-stage larvae. Larvae were cultured by using the Baermann method[18] over a period of 5 days, washed in distilled water, and concentrated to a volume of 10 mL by an 8-μm nitrocellulose filter (Millipore, Ballerica, MA). A total count of larvae was made and aliquots were diluted to achieve concentrations of 10, 5, and 1 per 50 μL of water. The number of larvae in each dilution was checked by microscopy and this step was replicated 10 times for each category. The mean ± SD of these counts were 10 ± 0.94 for aliquots containing 10 larvae, 4.7 ± 0.94 for aliquots containing 5 larvae, and 1.4 ± 0.69 for aliquots containing 1 larva.

Known number of *S. ratti* larvae (50 μL) was then spiked into 200 μL of human stool known to be negative by coprologic analysis (direct smear, APC, and Harada-Mori methods). These aliquots of human stool were then used to compare the sensitivity of different extraction methods. Each extraction method was used on four replicates of stool specimens containing each concentration of *S. ratti*.

DNA extraction comparison. Five techniques of DNA extraction were used for the isolation of DNA from stool specimens spiked with *S. ratti* third-stage larvae. These methods included four manual techniques. First, a modified QIAamp Tissue Kit spin column (QIAGEN, Hilden, Germany) with a preliminary step using polyvinylpolypyrolidone (Sigma, Steinheim, Germany) as described.[10] The three other manual techniques used were the PowerSoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA); the Ultra Clean Fecal DNA kit (Mo Bio Laboratories, Inc.), which were used according to the manufacturer's instructions; and the repeated bead beating plus column method (RBB+C), as described.[19] Finally, a semi-automated DNA extraction method using repeated bead beating combined with NucliSENS easyMAG (RBB+NeM) described for extraction of microbial DNA.[20] In this method, the repeated bead beating was followed by an automated extraction using a bio-robot with the standard reagents and protocols provided by the manufacturer (BioMérieux, Marcy l'Etoile, France). The last two methods used mechanical cell disruption by two rounds of bead beating as a pre-treatment step.[19,20] A DNA extraction control of 5 μL (catalogue no. Aus-99005S; Bioline, Alexandria, New South Wales, Australia) was added to the lysis buffer at the time of extraction, in accordance with the manufacturer's instructions.

Survey samples. A total of 160 stool samples were collected during a survey to identify *S. stercoralis*–infected persons in an urban slum of Dhaka, Bangladesh and cultured by using the Harada-Mori method within six hours of collection at the Institute of Epidemiology, Disease Control and Research,

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(Dhaka, Bangladesh). Ethical clearance was provided by the National Research Ethics Committee, Bangladesh Medical Research Council (Dhaka, Bangladesh; Reference BMRC/NREC/2007-2010/1256, January 20, 2009). This study was conducted in collaboration with the Institute of Epidemiology, Disease Control and Research. Because most participants were illiterate, consent and completion of a questionnaire were obtained verbally from each respondent at the time of collection. Participants who were infected with strongyloidiasis were offered albendazole, 400 mg/day, for 2 consecutive days (the only drug registered for treatment of this infection in Bangladesh).

Harada-Mori culture and survey sample extraction. Single stool specimens were collected from study participants and approximately two grams were used in each of triplicate Harada-Mori cultures, with a total amount of approximately 6 grams cultured per stool specimen.[18] Cultures were incubated at 25–28°C for one week and examined daily. Specimens were regarded as positive if at least one larva was identified in any of the triplicate culture vials. Microscopic examination of *Strongyloides* larva identifying specific morphologic structures[18] was used to confirm positive cultures. Stool samples were categorized into low (< 5), moderate (5–10), and high (> 10) parasite load according to the total number of larvae harvested from each stool specimen (triplicate vials). Survey samples were transported frozen in dry ice and stored at –20°C for approximately 12 months, until DNA extraction was performed. DNA from these survey samples was extracted by using the PowerSoil kit for further testing using real-time PCR.

Real-time PCR. The real-time PCR amplified a 101 basepair region of *S. stercoralis* 18S ribosomal DNA (Gene Bank accession no. AF279916) using the specific primers Stro18S-1530F (5′-GAATTCCAAGTAAACGTAAGTCAT TAGC-3′; Stro18S-1630R 5′-TGCCTCTGGATATTGCTCA GTTC-3′ and probe Stro18S-1586T (FAM-5′-ACACACCG GCCGTCGCTGC-3′-BHQ1).[10] The PCR conditions were optimized in our laboratory settings. The PCR mixture contained 0.3 μM of each primer, 0.1 μM of probe, 5 mM $MgCl_2$, and 0.1 mg/mL bovine serum albumin (Promega, Alexandria, New South Wales, Australia) and 10 μL of DNA template in a final volume of 50 μL of PCR buffer (HostarTaq master mixture; QIAGEN). One microliter of internal control reaction mixture was introduced per reaction according to manufacturer's instructions to check for inhibition of the assay. The reaction mixture was amplified on a Corbett Rotor-Gene 6000 apparatus (QIAGEN) for 15 minutes at 95°C, followed by 40 cycles for 15 seconds at 95°C and 30 seconds at 60°C. Amplification, detection, and data analysis were performed by using Corbett Rotor-Gene 6000 Series Software version 1.7 (QIAGEN). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by using SYBR safe stain. The correct product was confirmed for every amplified sample by sequence analysis and the National Center for Biotechology Information (Bethesda, MD) basic local alignment search tool alignment.

Sensitivity and specificity of real-time PCR. Serial dilutions were performed on the DNA from single *S. ratti* larva spiked into stool, which was extracted by using the PowerSoil kit. Dilutions were 10-fold to a concentration of 10^{-3} and used to determine the sensitivity of the real-time PCR. Specificity of the assay was assessed by using a panel of DNA extracted from *Staphylococcus aureus*, *Clostridium difficile*, *Enterococcus faecalis*, *Mycobacterium tuberculosis*, *Shigella sonnei*, *Campylobacter jejuni*, *Bacteroides fragilis*, *Penicillium chrysogenum*, *Cryptococcus neoformans*, *Candida albicans*, *Candida tropicalis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Dientamoeba fragilis*, *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Blastocystis hominis*, *Giardia duodenalis*, *Trichostrongylus vitrinus*, *Ascaris lumbricoides*, *Taenia saginata*, *Enterobius vermicularis*, *Ancylostoma caninum*, *Fasciola hepatica*, and *Angiostrongylus cantonensis*. In addition, DNA from 30 fresh human stool samples that were negative by direct smear microscopy and APC culture were extracted by using the PowerSoil kit and tested in real-time PCR. Ethics clearance for this collection was provided by the Human Research Ethics Committee, Sydney West Area Health Service (HREC2010/6/5.1 (3169) QA.

RESULTS

Comparison of different extraction methods in real-time PCR. *Strongyloides ratti* DNA extracted from spiked samples using the five methods was detected by real-time PCR (Table 1). *Strongyloides ratti* DNA extracted with the PowerSoil kit was detected by real-time PCR with the lowest threshold cycle (C_t) values, regardless of the number of spiked larvae. A similar result was achieved when the modified QIAamp Tissue Kit spin column was used, but slightly higher C_t values were observed. DNA extracts by using the Ultra Fecal DNA kit, the RBB+C method, and the RBB+NeM method gave reduced detection rates and higher C_t values with the real-time PCR.

Sensitivity and specificity. Serial dilutions were made of the stool samples spiked with a single *S. ratti* larva and extracted by using the PowerSoil kit. The limit of detection for all four replicates was to a 10^{-2} dilution. The use of the synthetic DNA internal control did not affect the sensitivity of the assay. DNA from a series of bacteria (n = 7), fungi (n = 9), and parasites (n = 12) were negative in the real-time PCR. The 30 control stools, which were negative by microscopy and APC, were negative by real-time PCR.

Comparison of culture and real-time PCR from surveyed stool samples. Forty-one of 160 stool samples were positive in at least one of the triplicate Harada-Mori culture vials, corresponding to a prevalence rate of 25% in the surveyed samples. Fourteen of the 41 culture-positive stools were positive by real-time PCR. All four stool samples with moderate worm burdens and all five stool samples with high worm burdens were successfully identified by real-time PCR. However, of those with a low worm burden, only 5 (15%) of 32 culture-positive stools were positive by real-time PCR (Table 2). In

TABLE 1

Comparative C_t values in real-time PCR by using five extraction methods for isolation of *Strongyloides* DNA in stool samples*

Extraction method	No. *S. ratti* third-stage larvae spiked in stool (no. positive/no. tested)		
	10	5	1
PowerSoil kit	25 (4/4)	28 (4/4)	28 (4/4)
Fecal DNA kit	27 (4/4)	27 (4/4)	30 (3/4)
QIAamp Tissue kit (modified)	28 (4/4)	30 (4/4)	30 (4/4)
RBB+C	34 (2/4)	36 (1/4)	36 (2/4)
RBB+NeM	31 (4/4)	32 (4/4)	32 (3/4)

*PCR = polymerase chain reaction; C_t = cycle threshold; RBB+C = repeated bead beating plus column method; RBB+NEM = semi-automated DNA extraction method using repeated bead beating combined with NucliSENS easyMAG. Values in parentheses are number of real-time PCRs detected in quadruplicate samples,

TABLE 2

Comparison of total number of larvae detected by Harada-Mori culture and by real-time PCR*

Stool samples	No worms/culture	No. positive cultures	No. real-time PCR positive (C_t range)
119	None	0	1 (32)
32	Low (1–5)	32	5 (36–38)
4	Moderate (5–10)	4	4 (30–35)
5	High (> 10)	5	5 (23–29)
Total = 160		41	15

*PCR = polymerase chain reaction; C_t = cycle threshold.

addition, one culture-negative stool sample was positive by real-time PCR, giving a total of 15 real-time PCR-positive stool samples. Specificity of the real-time PCR was > 99% when compared with Harada-Mori culture.

DISCUSSION

One semi-automated and four manual extraction methods were compared by using parallel DNA extractions of *S. ratti*–spiked stool samples and evaluated with a real-time PCR. Of the methods tested, the PowerSoil kit was found to be the best in terms of real-time PCR sensitivity and ease of use. The real-time PCR amplified DNA extracted with the PowerSoil kit at least 2–3 cycles earlier than other extraction procedures. Detectable DNA was present in a 10^{-2} dilution of extract from one larva spiked into stool to a volume of 250 μL. The results using the modified QIAamp Tissue Kit had slightly later C_t values, indicating less DNA template present compared with the PowerSoil kit.[10,12] The PowerSoil kit also had the advantage of shorter incubation times than the modified QIAamp Tissue Kit. The detection rates were lower with the Ultra Fecal DNA isolation kit, the RBB+C method, and the RBB+NeM method.

In our study, real-time PCR detected 100% of survey stool samples identified with high and moderate worm burdens, but detected only 15% of culture-positive samples with low larval numbers (< 5/culture). Overall, the real-time PCR detection rate was 33% compared with culture. Many of the culture-positive samples had relatively low larval numbers, which is consistent with chronic infection.[1] Surveyed stool samples were collected from asymptomatic persons living in a slum with a high risk of exposure to *S. stercoralis*. The low worm numbers (< 5) were found in triplicate Harada-Mori cultures using six grams of stools, compared with approximately 250 mg used for stool DNA extraction.

The sensitivity of PCRs has been shown to be reduced with low parasite burden in a model where rats were infected with *S. venezuelensis*[10] and in a clinical study in Ghana.[21] In the clinical study, 54 samples were positive for *S. stercoralis* with the Baermann technique and duplicate stool cultures.[10] Of the positive samples 45 (83%) were positive by real-time PCR. However, in instances in which there was a lower burden of infection and *S. stercoralis* larvae were detected in only one of duplicate cultures, only 5 (23%) of 21 samples were positive by real-time PCR. This subset also showed correspondingly high C_t values. In our study, samples with low larval numbers also showed higher C_t values. All culture-positive samples with high and moderate parasitic numbers showed low C_t values, indicating a higher intensity of infection.

One of the Harada-Mori culture-negative samples was positive by real-time PCR, corresponding to a specificity > 99%. This result may represent a false-positive result. As with other products of PCRs, the DNA sequence from the culture-negative sample matched that of *S. stercoralis*. This finding may indicate that non-viable larvae were present at the time of culture, leading to a negative result.

The real-time PCR results for stool spiked with *S. ratti* and extracted with the PowerSoil kit showed a high degree of sensitivity, with a 10^{-2} dilution of an extract from a single larva being positive. However, results from 160 samples collected in Bangladesh showed a lower sensitivity. Explanations for this discrepancy include low larval numbers, the difference in the volume of stool tested with culture compared with that tested by PCR, variable distribution of larvae in specimens, and that the surveyed specimens were frozen for > 12 months before DNA extraction, which may have led to degradation.

Real-time PCR using the PowerSoil DNA extraction kit was suitable for rapid detection of *S. stercoralis* in a clinical laboratory in persons with moderate-to-high larval number. However, in this study, real-time PCR was relatively insensitive when larval numbers were low, as in chronic infection. Storage of these survey stool samples at –20°C for 12 months may have contributed to DNA degradation and thus hamper the clinical interpretation of this study. Another study is required to draw a conclusion on how possible degradation of DNA in stool over time limits this diagnostic test. However, in the clinical setting, specimens would be tested within days of receipt and not be subjected to prolonged storage conditions. Furthermore, sensitivity of this real-time PCR could be improved either with DNA extraction from a larger amount stool or by testing multiple stool samples.

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