Transactions of the Royal Society of Tropical Medicine and Hygiene (2009) **103**, 342—346

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Molecular diagnosis of *Strongyloides stercoralis* in faecal samples using real-time PCR

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Received 29 September 2008; received in revised form 26 November 2008; accepted 1 December 2008
Available online 4 February 2009

KEYWORDS
Strongyloides stercoralis;
Strongyloidiasis;
Parasitic intestinal disease;
Diagnosis;
Baermann sedimentation;
Real-time PCR

Summary A real-time PCR method targeting the small subunit of the rRNA gene was developed for the detection of *Strongyloides stercoralis* DNA in faecal samples, including an internal control to detect inhibition of the amplification process. The assay was performed on a range of well-defined control samples ($n = 145$), known positive faecal samples ($n = 38$) and faecal samples from a region in northern Ghana where *S. stercoralis* infections are highly endemic ($n = 212$), and achieved 100% specificity and high sensitivity. The use of this assay could facilitate monitoring the prevalence and intensity of *S. stercoralis* infections during helminth intervention programs. Moreover, the use of this assay in diagnostic laboratories could make the introduction of molecular diagnostics feasible in the routine diagnosis of *S. stercoralis* infections, with a two-fold increase in the detection rate as compared with the commonly used Baermann sedimentation method.

1. Introduction

Laboratory diagnosis of strongyloidiasis is primarily based on the detection of *Strongyloides stercoralis* larvae by microscopic examination of stool samples. The number of larvae present is very small, especially in chronic infections, and even using formalin-ether concentration, the Baermann method or coproculture the detection rate is low and multiple samples have to be examined to achieve adequate sensitivity.[1—5]

Several immunodiagnostic assays have been published, with variable sensitivity and specificity depending on the antigen preparation used, the immunoglobulin isotypes and the population tested. The sensitivity of serology is good in individuals with chronic infection but is lower in those who become infected after travelling to endemic areas.[6]

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0035-9203/$ — see front matter © 2008 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.trstmh.2008.12.001

Although, in endemic areas, serology responds well after successful treatment,[7] the technique has been reported to lack specificity in endemic areas and further evaluation is needed.[8]

Recently, detection of parasite DNA in faecal samples using real-time PCR proved to be a sensitive and specific method for the diagnosis of intestinal protozoan and helminth infections.[9–11]

In the present study a real-time PCR was developed for the specific detection of *S. stercoralis* DNA in faecal samples. Additionally, an internal control for the detection of possible inhibition of the amplification by faecal contaminants was included in the assay. The performance of the PCR was evaluated using a large range of control DNA and stool samples.

2. Materials and methods

The reason for the survey and the procedure of sample collection were explained to all adult participants and parents of children, as well as the community leaders, before requesting stool samples. Oral informed consent was obtained from all participating individuals.

2.1. Controls

To develop the real-time PCR assay, control DNA was obtained from L3 larvae of *S. stercoralis* from coproculture. Specificity of the PCR was tested using DNA isolated from adult worms of *Ascaris lumbricoides*, *Trichuris trichiura*, *Schistosoma mansoni*, *Necator americanus* and *Oesophagostomum bifurcum* and L3 larvae of *Ancylostoma duodenale* as template. In addition, DNA from *Entamoeba histolytica*, *E. dispar*, *Giardia lamblia*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* was tested in the PCR.[12] The specificity of the assay was also tested on DNA obtained from 17 different bacterial and yeast cultures: *Bacillus cereus*, *Campylobacter jejuni*, *Campylobacter upsaliensis*, *Candida albicans*, *Escherichia coli* O157, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus* and *Yersinia enterocolitica*. Furthermore, 80 stool samples from different patients were tested in which *E. histolytica* ($n = 20$), *E. dispar* ($n = 20$), *G. lamblia* ($n = 20$) or *C. parvum*/*C. hominis* ($n = 20$) had been detected by microscopy and confirmed by specific PCR.[13,14] The specificity of the PCR assay was also tested on DNA extracts from 40 unpreserved stool samples with a negative result in microscopy using the Baermann technique, formalin-ether sedimentation or modified acid-fast staining, and a negative *Giardia* antigen test. Of these negative samples, two subsequent stool samples from these patients remained negative by all techniques.

For a preliminary evaluation of the PCR, DNA isolated from 38 human faecal samples from La Merced, Peru was used. These faecal samples originated from individual *S. stercoralis* cases in which Baermann sedimentation showed first-stage larvae (L1 s) of *S. stercoralis*. In 13 of these samples L1 larvae of *S. stercoralis* were also seen in a direct smear.

2.2. Field samples

DNA isolated from stool samples ($n = 212$) collected in northern Ghana was used to evaluate the sensitivity of the real-time PCR compared with Baermann sedimentation and coproculture. These stool samples were collected during a cross-sectional survey in a number of small communities situated in northern Ghana in which a high prevalence of *S. stercoralis* infection was found in previous studies.[15] Samples were selected for DNA isolation and PCR based on the availability of results from Baermann sedimentation tests and duplicate coprocultures. Coproculture and differentiation of *S. stercoralis* from other nematode larvae was carried out as previously described.[16,17]

Faecal samples were transported to Leiden for DNA isolation, either frozen or suspended in ethanol, and stored at room temperature.

2.3. DNA isolation

Approximately 100 mg unpreserved faeces were suspended in 200 μl PBS containing 2% polyvinylpolypyrolidone (PVPP; Sigma, Steinheim, Germany). After heating for 10 min at 100 °C suspensions were treated with sodium dodecyl sulphate-proteinase K for 2 h at 55 °C. DNA was isolated using QIAamp Tissue Kit spin columns (QIAgen, Hilden, Germany).[18] Within the isolation lysis buffer, 10^3 PFU/ml phocine herpes virus 1 (PhHV-1) was added to serve as an internal control.[19] The amplification was considered to be hampered by faecal inhibitory factors if the expected cycle threshold (Ct) value of 33 in the PhHV-specific PCR was increased by more than 3.3 cycles.

2.4. Real-time PCR

Three sets of *S. stercoralis*-specific primers and detection probes were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) from the cytochrome c oxidase subunit I gene, a *S. stercoralis*-specific repeated sequence and the 18S rRNA gene sequence, respectively (GenBank accession nos. **U57039**, **AY028262** and **AF279916**). DNA sequences and further specifications of the *S. stercoralis*-specific primers and probe sets are given in Table 1. For internal control, PhHV-1-specific primers and probe set consisted of forward primer PhHV-267 s, reverse primer PhHV-337as and the specific double-labelled probe PhHV-305tq (Biolegio, Nijmegen, The Netherlands).[19] An NCBI BLAST search was used to test the theoretical specificity of the primers and probe. A 10-fold dilution series of *S. stercoralis* control DNA was used to optimize the real-time PCR and was tested with and without the presence of internal control DNA to estimate the latter's influence.

For DNA amplification, 5 μl of DNA extracted from stool specimens was used as a template in a final volume of 25 μl with PCR buffer (HotstarTaq master mix, QIAgen), 5 mM $MgCl_2$, 2.5 μg bovine serum albumin (Roche Diagnostics, Almere, The Netherlands), 1.5 pmol of each *S. stercoralis*-

Table 1 Oligonucleotide primers and detection probes for real-time PCR on different targets for the detection of *Strongyloides stercoralis* DNA

Target (GenBank accession no.) Oligo name	Oligonucleotide sequence	Expected product size
S. stercoralis-specific repeated sequence (**AY028262**)		
Stro F	5′-TCCAGAAAAGTCTTCACTCTCCAG-3′	96 bp
Stro R	5′-TGCGTTAGAATTTAGATATTATTGTTGCT-3′	
Strongy	FAM-5′-TCAGCTCCAGTTGAACAACAGCCTCCAA-3′-BHQ1	
S. stercoralis cytochrome c oxidase subunit I gene (**U57039**)		
StroCyt360F	5′-CATCCTGGTTCTAGTGTTGATTTGG-3′	121 bp
StroCyt480R	5′-GAGAAACAGAACTAGAACGCAAATTTT-3′	
StroCyt399-MGB	FAM-5′-CATCTTTCTGGTATTAGTTCTAT-3′-MGB-NFQ	
S. stercoralis 18S rRNA gene (**AF279916**)		
Stro18S-1530F	5′-GAATTCCAAGTAAACGTAAGTCATTAGC-3′	101 bp
Stro18S-1630R	5′-TGCCTCTGGATATTGCTCAGTTC-3′	
Stro18S-1586T	FAM-5′-ACACACCGGCCGTCGCTGC-3′-BHQ1	

BHQ: black hole quencher; MGB: minor groove binder; NFQ: non-fluorescent quencher.

Table 2 Comparison of Baermann sedimentation, coproculture and real-time PCR for the detection of *Strongyloides stercoralis* in human stool samples ($n = 212$) from northern Ghana

n	Microscopy			PCR	
	Baermann	Culture A	Culture B	Positive	Negative
12	+	+	+	12	0
2	+	–	+	2	0
8	+	–	–	5	3
11	–	+	+	9	2
5	–	+	–	1	4
16	–	–	+	4	12
158	–	–	–	12	146

specific primer, 3.75 pmol of each PhHV-1-specific primer, 2.5 pmol of *S. stercoralis*- and PhHV-1-specific double-labelled probes. Amplification consisted of 15 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification, detection and data analysis were performed with the Applied Biosystems 7500 Real-Time PCR system and sequence detection software version 1.2.2.

3. Results

In the NCBI BLAST search, primers and probe sets on the *S. stercoralis*-specific repeated sequence, the cytochrome c oxidase subunit I gene and the 18S rRNA gene sequence showed 100% specificity for *S. stercoralis*. The forward primer on the 18S rRNA gene sequence also showed 100% homology with other *Strongyloides* species.

The real-time PCRs were optimized using a 10-fold dilution series of *S. stercoralis* positive-control DNA. In the 100-fold dilution of the control DNA, threshold cycles of 23.7, 26.9 and 29.5 were detected using primers and probes designed from the cytochrome c oxidase subunit I gene, the *S. stercoralis*-specific repeated sequence and the 18S rRNA gene sequence, respectively. The Ct values obtained from testing the dilution series of *S. stercoralis* DNA in both the individual *S. stercoralis* assay and the multiplex assay with the internal control showed no systematic deviation in amplification curves, and the same analytical sensitivity was achieved. The individual performance of the assays was not influenced by the presence of DNA from the internal control.

The specificity of the real-time *S. stercoralis* 18S PCR was evaluated using a range of parasite and bacterial control DNA ($n = 25$), 80 DNA extracts derived from faeces positive for *E. histolytica*, *E. dispar*, *G. lamblia* or *C. parvum*/*C. hominis*, and 40 DNA extracts derived from faeces of individuals with no known history of parasitic infections. No amplification of *S. stercoralis*-specific DNA was detected in any of these 145 samples, only the amplification of the internal control was detected at the expected cycle threshold of approximately 33.

Performance of the real-time PCR was first evaluated using DNA isolated from 38 stool samples in which the Baermann sedimentation test showed L1 larvae of *S. stercoralis*. *Strongyloides stercoralis*-specific amplification was detected in 12 of 13 (92%) samples in which *S. stercoralis* was detected by microscopic examination of a direct smear and in 18 of 25 (72%) samples in which *S. stercoralis* was not

detected in the direct smear but was detected by Baermann sedimentation.

Stool samples ($n = 212$) from northern Ghana were used for a more elaborate evaluation of the real-time PCR. L1 larvae of *S. stercoralis* were demonstrated in the Baermann sediment in 22 of 212 (10.4%) samples. L3 larvae of *S. stercoralis* were seen in the first and/or the second coproculture in 46 of 212 (21.7%) samples. In total, 54 samples (25.5%) showed L1 s and/or L3 larvae.

Table 2 summarizes the comparison between microscopy and real-time PCR. *Strongyloides stercoralis*-specific amplification was detected in 45 (21.2%) samples. In 19 of 22 (86.4%) faecal samples with *S. stercoralis* L1 larvae observed in the Baermann test, *S. stercoralis*-specific amplification was detected with Ct 22.0—35.9 (median 28.1). In those cases in which *S. stercoralis* was detected in both of the duplicate coprocultures, *S. stercoralis*-specific amplification was detected in 21 of 23 (91.3%) samples (Ct median 28.6, range 23.3—35.0). *Strongyloides stercoralis*-specific amplification was demonstrated (Ct median 36.2, range 26.0-39.7) in 5 of 21 faecal samples of those cases in which *S. stercoralis* L3 larvae were detected in one of the duplicate coprocultures only. Also, *S. stercoralis*-specific amplification was detected in 12 of 158 DNA samples from stools in which no *S. stercoralis* L1 or L3 larvae were found (Ct median 36.6, range 27.9—40.6).

The PhHV-1 internal control was amplified within the correct Ct range in all stool samples.

4. Discussion

In the present study a *S. stercoralis* real-time PCR was developed and achieved 100% specificity using a large range of control DNA and stool samples. The primer and probe set from the 18S rRNA gene sequence was 10-fold to 100-fold more sensitive than the PCR designed from the cytochrome c oxidase subunit I gene or the *S. stercoralis*-specific repeated sequence.

In previous studies on the application of real-time PCR for the specific detection of hookworm and *Schistosoma* infections it was shown that the Ct values reflecting the amount of parasite-specific DNA in the sample correlate with the intensity of infection.[9,11] In the present study, the low intensity of the *S. stercoralis* infections was reflected in the discrepant results between the Baermann test and/or the duplicate coprocultures in 42 of 54 microscopy-positive samples. This phenomenon is also shown in the lower detection rate and the higher median Ct value of the cases in which the Baermann test did not show L1 larvae and only one of the duplicate cultures was positive.

The false-negative real-time PCR results were not caused by inhibition of the amplification by faecal components. A possible explanation could be that the amount of stool used in a Baermann test or duplicate coproculture is approximately 40 times greater than that used for DNA isolation. Also, the possibility of misidentification of larvae by microscopy cannot be excluded, especially as these samples were collected in a region with high prevalence and intensity of hookworm infections.[20]

The excretion and distribution of parasite DNA in faeces is expected to be less variable than the number of eggs. This has already been shown for the amplification of parasite-specific DNA compared with the microscopic detection of cysts or oocysts from *G. lamblia* and *Cryptosporidium* and eggs of *Schistosoma*.[9,14] Further studies to compare the outcome of Baermann sedimentation, coproculture and real-time PCR in three consecutive samples from the same subject are planned.

The collection of stool samples in ethanol allows storage at room temperature and transportation to central research centres with facilities for real-time PCR, simplifying the complex organization of labour-intensive field studies. Combined with other parasitic targets such as *N. americanus*, *A. duodenale*,[11] *A. lumbricoides*[21] and *T. trichiura* this *S. stercoralis* PCR could be used for epidemiological research and monitoring of the species-specific effect of intervention programs for the control of soil-transmitted helminths.

In most endemic countries this procedure will not be feasible as a routine diagnostic tool in clinical settings where laboratory facilities are often limited. In routine diagnostic laboratories in industrialized countries, however, molecular diagnostics are used for the laboratory diagnosis of an increasing number of infectious targets.[10,22] In such settings the *S. stercoralis* real-time PCR could be a useful alternative to the commonly used Baermann method, offering a two-fold increase in the detection rate.

Authors' contributions: JJV and LvL were responsible for the design of the study; JJV designed the real-time PCRs; MC, KP, JZ, EATB and AMP were responsible for sample and microscopic data collection; JJV was responsible for the DNA isolation and real-time PCR; JJV and LvL were responsible for data analysis and interpretation of the data; JJV wrote the paper and all authors made significant comments. All authors read and approved the final manuscript. JJV is guarantor of the paper.

Acknowledgements: To the field team workers for their help in sample collection and microscopic analysis and to all students involved. The Ghana Health Service in Bolgatanga and Tamale is gratefully acknowledged for its support.

Funding: Sample collection in Peru was supported by the Directorate-General for Development Cooperation (DGDC) of the Belgian Government through a framework agreement with the Institute of Tropical Medicine of Antwerp. Financial support was provided by the Dutch Foundation for the Advancement of Tropical Research WOTRO-NWO and the *Oesophagostomum* Research Intervention Project in Northern Ghana.

Conflicts of interest: None declared.

Ethical approval: The study was given ethical approval by the Ghanaian Ministry of Health.

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Transactions of the Royal Society of Tropical Medicine and Hygiene

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Corrigendum

Corrigendum to "Molecular diagnosis of *Strongyloides stercoralis* in faecal samples using real-time PCR" [Transactions of the Royal Society of Tropical Medicine and Hygiene (2009) 103, 342–6]

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ARTICLE INFO

Article history:
Available online 17 March 2010

In the second paragraph of the results section of this paper we indicated that "In the 100-fold dilution of the control DNA, threshold cycles of 23.7, 26.9 and 29.5 were detected using primers and probes designed from the cytochrome c oxidase subunit I gene, the *S. stercoralis*-specific repeated sequence and the 18S rRNA gene sequence, respectively." The best target and the worst target were inadvertently swapped over and this text should read 'In the 100-fold dilution of the control DNA, threshold cycles of 23.7, 26.9 and 29.5 were detected using primers and probes designed from the 18S rRNA gene sequence, the *S. stercoralis*-specific repeated sequence and the cytochrome c oxidase subunit I gene, respectively.'

We apologise for this error and are grateful to those careful readers who wanted to implement the assay and contacted us to ask which primer and probe set they should use.

DOI of the original article:10.1016/j.trstmh.2008.12.001.
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doi:10.1016/j.trstmh.2010.02.010