

Novel real-time PCR for the universal detection of *Strongyloides* species

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Strongyloidiasis is a neglected disease that is prevalent mainly in tropical and subtropical regions. It is caused by intestinal nematodes of the genus *Strongyloides*. Due to the rise in worldwide travel, infections are increasingly encountered in non-endemic regions. Diagnosis is hampered by insensitive and laborious detection methods. A universal *Strongyloides* species real-time PCR was developed with an internal competitive control system. The 95% limit of detection as determined by probit analysis was one larva per PCR equivalent to 100 larvae per 200 mg stool. The assay proved to be 100% specific as assessed using a panel of parasites and bacteria and thus might be useful in the diagnostic setting as well as for *Strongyloides* research.

INTRODUCTION

Nematodes of the genus *Strongyloides* are widely distributed in tropical and subtropical countries and pose a significant health threat to humans (Bethony *et al.*, 2006). An estimated 30–100 million people are infected worldwide. In light of frequent international travel and immigration, strongyloidiasis is increasingly encountered in travel clinics in non-endemic regions (Nuesch *et al.*, 2005). At least two species are known to infect humans, *Strongyloides stercoralis* and *Strongyloides fuelleborni*. *Strongyloides ratti*, which naturally occurs in rats, is widely used as an experimental infection model to study strongyloidiasis (Viney, 1999). In immunocompetent humans, *Strongyloides* infection usually remains asymptomatic or is associated with mild and nonspecific symptoms only (Olsen *et al.*, 2009). Due to the ability to replicate within the host, chronic auto-infection may occur and long-term persistence of *Strongyloides* infection over decades has been described (Igual-Adell *et al.*, 2004). In immunocompromised hosts, however, *S. stercoralis* infections usually present as severe hyperinfection syndromes with mortality rates of up to 87% (Marcos *et al.*, 2008).

Standard diagnostic procedures in humans comprise concentration and stool culture methods (Siddiqui & Berk, 2001). A combination of these methods yields highest sensitivity especially if multiple stool samples are examined. However, this procedure is rather laborious and time-consuming. Several serum antibody detection methods do exist with variable sensitivities and specificities depending on the antigen used (Ravi *et al.*, 2002), the immunoglobulin isotypes or the study population (Silva *et al.*, 2003;

Rodrigues *et al.*, 2007). As a major drawback of serological tests, considerable cross-reactivity between the various nematode species has been observed. Additionally, antibody detection methods lack sensitivity especially in returning travellers (Sudarshi *et al.*, 2003).

As DNA detection becomes increasingly utilized in the diagnosis of parasite infection, this method might have the potential to overcome the problems in diagnosing strongyloidiasis (ten Hove *et al.*, 2009). A novel real-time PCR detection system recently proved successful in a small pilot study but data on its analytical sensitivity are lacking (Verweij *et al.*, 2009). In this communication, we report on the development of a real-time PCR assay for sensitive detection of *Strongyloides* species DNA in stool samples which can be used both in research and in the diagnostic setting. The assay was thoroughly evaluated from a technical point of view. In addition, an internal control system was established to monitor the influence of possible inhibitory activity.

METHODS

Parasite isolates. The *S. ratti* infection cycle established in our laboratory was originally provided by Dr G. Pluschke (Swiss Tropical Institute, Basel). Wistar rats were used to maintain the cycle by serial passage as described by Viney & Lok (2007). In brief, Wistar rats were infected subcutaneously with 2500 L3 *S. ratti* larvae. Faeces were collected from day 1 until day 40. Approval was obtained from the animal protection board of the City of Hamburg. All faecal pellets were collected daily from day 1 until day 40, suspended in PBS with charcoal and incubated for 5 days at 26 °C (Viney & Lok, 2007). Infective larvae were collected by the standard Baermann extraction technique (García & Bruckner, 2001), centrifuged, resuspended and counted microscopically.

DNA preparation from stool samples. For isolation of nucleic acids from stool samples, the QIAmp DNA stool kit (Qiagen) was

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Abbreviations: FRET, fluorescence energy transfer; LOD, limit of detection.

used according to the manufacturer's instructions. Two hundred milligrams of human stool or faeces from infected as well as uninfected rats was used.

Real-time PCR. A novel real-time PCR for *Strongyloides* species targeting the 28S rRNA gene was developed (Fig. 1). Using BLAST search software (<http://ncbi.nlm.nih.gov/BLAST/>), all available *Strongyloides* species 28S rRNA sequences were aligned. Upon manual inspection, a conserved region was identified fitting the demands of fluorescence energy transfer (FRET)-based real-time PCR assays. A 20 µl reaction contained 2 µl PCR buffer (Applied Biosystems), 200 µM of each dNTP (Applied Biosystems), 2.5 mM MgCl₂, 750 nM StroS (5'-TTA-GAGTCGTGTTGCTTGAA-3'), 1 µM StroAS (5'-GTGCAACTGG-CTCTGTATGC-3'), 50 nM StroP3 (5'-CTGTGAAGGAAAATTGCA-AAGTACTCCGGA-FAM-3'), 200 nM StroP4 (5'-LC640-GAGAGTT-AAAGAGGACGTGAAACCGATACG-PH-3'), 200 nM StroP5 (5'-LC705-ATCGTTCGTTGAGCGATTAGCAGTT-PH-3'), 0.8 µg BSA and 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The amplified fragment length was 180 bp. Probe StroP3 was labelled with fluorescein at its 3'-end and probe StroP4 was labelled with LightCycler Red 640 at its 5'-end and phosphorylated at the 3'-end. Correspondingly, StroP5 was labelled with LightCycler Red 705 at its 5'-end and phosphorylated at the 3'-end. Two microlitres of DNA solution was added to each reaction. Thermal cycling in a LightCycler 1.5 Instrument (Roche Diagnostics) involved denaturation at 95 °C for 15 min, followed by 45 cycles of 95 °C for 5 s, 50 °C for 20 s and 72 °C for 30 s. Fluorescence was measured during the 50 °C step on detection channel F2 (640 nm) and divided by fluorescence measured on channel F1 (530 nm) for normalization. The internal control signal was measured in channel F3 (705 nm) of the LightCycler instrument and divided by fluorescence measured on channel F1 (530 nm).

Construction of DNA standard. The 180 bp real-time amplicon of the 28S rRNA gene of *S. ratti* was ligated into pCR2.1 plasmid vector and cloned into *Escherichia coli* using the pCR 2.1 TOPO TA cloning kit (Invitrogen) as described by Panning *et al.* (2008). Resulting plasmids (pStrowild) of the correct length were purified by means of a QIAprep Spin Miniprep kit (Qiagen) and DNA concentration was spectrophotometrically measured. Sequence integrity was checked by sequencing on a CEQ 8000 Genetic Analysis System (Beckmann Coulter).

Construction of internal control. A competitive internal control system was incorporated into the assay by gene splicing and overlap extension as described by Panning *et al.* (2008). In brief, using primers IcS (5'-ATCGTTCGTTGAGCGATTAGCAGTTGTTATGGA-AGCATAACAGAGCCAGTTGCAC-3') and IcAs (5'-AACTGCTAAT-CGCTCAACGAACGATTCTCCGGAGTACTTTGCAATTTTCCTTC-3'), an alternative probe binding site was introduced into pStrowild yielding pStroIC. A probe alternatively labelled with dye LC705 (StroP5) was used for detection of pStroIC in a separate channel of the LightCycler instrument.

Probit analysis. The lower limit of detection (LOD) was determined by probit analysis of multiple parallel amplifications (×5) of stool spiked with plasmid pStrowild as described by Panning *et al.* (2008). In addition, different input numbers of microscopically determined *S. ratti* larvae were spiked in human stool samples from healthy German volunteers which tested negative for parasites upon microscopy. All samples were correspondingly tested in multiple parallel amplifications. Statgraphics Plus, version 5 (Manugistics), was used for statistical analysis.

Cross-reactivity panel. DNA from the following parasites was tested with the novel real-time PCR: *Ancylostoma duodenale*, *Necator americanus*, *Trichuris trichiura*, *Ascaris suum*, *Trichinella spiralis*, *Toxocara canis*, *Wuchereria bancrofti*, *Loa loa*, *Dirofilaria immitis*,

Organism	StroS TTAGTCGTGTTGCTTGAA//	StroP3 CTGTGAAGGAAAATTGCAAGTACTCCGGA//	StroP4 GAGAGTTAAGAGACGTGAAACCGATACG//	StroAS GTGCAACTGGCTCTGTATGC	GenBank accession no.
<i>S. stercoralis</i>	---	---	---	---	AY 294186
<i>S. stercoralis</i>	---	---	---	---	DQ 145661
<i>S. stercoralis</i>	---	---	---	---	U 39489
<i>S. stercoralis</i>	---	---	---	---	U 38855
<i>S. fueleborni</i>	---	---	---	---	AB 272235
<i>S. fueleborni</i>	---	---	---	---	U42595
<i>S. ratti</i>	---	---	---	---	U 39490
<i>S. robustus</i>	---	---	---	---	AB 272233
<i>S. robustus</i>	---	---	---	---	AB 272232
<i>S. calloscinreus</i>	---	---	---	---	AB 272231
<i>S. calloscinreus</i>	---	---	---	---	AB 272230
<i>S. calloscinreus</i>	---	---	---	---	AB 272229
<i>S. procyonis</i>	---	---	---	---	AB 205054
<i>Ascaris I.</i>	-G-----G-----C-C-	-C---G---G-----A--TT-A	---C---G---G-----T-GA	---TTCGT-AA---A-CC-	U 94751
<i>Ascaris I.</i>	-G-----G-----C-C-	-C---G---G-----A--TT-A	---C---G---G-----T-GA	---TTCGT-AA---A-CC-	AY 210806
<i>Necator a.</i>	-G-----G-----A--	-C---C---A-----T-C-GGAG	---C---A-----A-----	ACTACGTCAA---C-CC-G	AM 039740

Fig. 1. Aligned binding region of oligonucleotides within 28S rRNA gene sequences available for *Strongyloides* species, *Ascaris lumbricoides* (*Ascaris I.*) and *Necator americanus* (*Necator a.*). Oligonucleotide descriptions are indicated above the alignment. The antisense oligonucleotide (StroAs) is depicted as the reverse complementary strand. Organism designations are given on the left, GenBank accession numbers on the right.

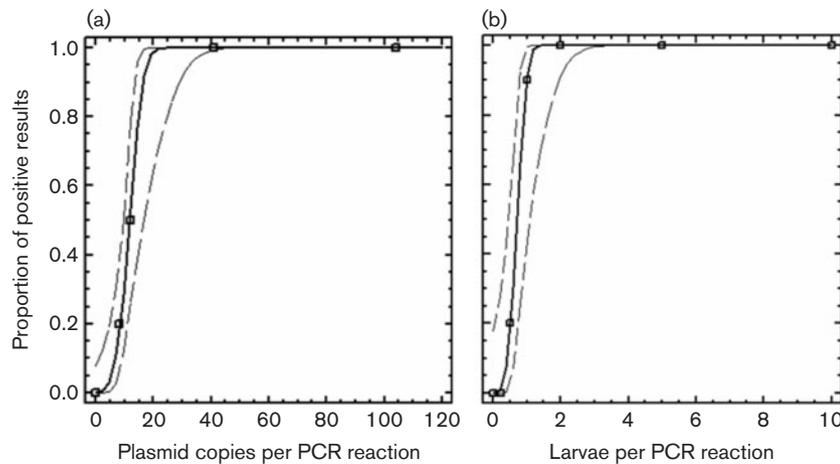


Fig. 2. Probability of achieving a positive test result (*y*-axis), depending on DNA copy input number per PCR (*x*-axis). Each datum point represents the rate of positive results in five replicate tests per concentration. Broken lines indicate 95% confidence intervals. (a) Plasmid pStrowild spiked in human stool samples; (b) microscopically counted larvae spiked in human stool samples.

Echinococcus granulosus and *Schistosoma mansoni*. In addition and to test for possible cross-reactivity, nucleic acid samples from the following panel of common bacteria and viruses were used (Panning *et al.*, 2007): Epstein–Barr virus (patient isolate), hepatitis B virus (INSTAND ref. material 11019), hepatitis C virus (1st International WHO NAT standard), human cytomegalovirus (INSTAND ref. material 15005), herpes simplex virus type 1 (INSTAND ref. material 13017), human immunodeficiency virus type 1 (NL-43), *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (in-house reference strain), *Staphylococcus aureus* (ATCC 13565) and *Streptococcus pyogenes* (ATCC 19615). In addition, DNA from the protozoan parasite *Plasmodium falciparum* (patient isolate) was used.

Clinical specimens. Thirty-five frozen stool samples from patients visiting the outpatient clinic of the Bernhard Nocht Institute for Tropical Medicine for reasons other than suspected strongyloidiasis were analysed retrospectively. All stool samples were negative for parasites upon microscopic inspection. Concomitantly, ten stool samples from ten patients with microscopic detection of *Entamoeba dispar* cysts, ten patients with *Giardia lamblia* and three patients with *Enterobius* species eggs as well as one patient positive for *Ascaris suum* and one patient with hookworms, were tested. Residual testing of patient samples was approved by the institutional review board of the Bernhard Nocht Institute.

RESULTS AND DISCUSSION

A novel real-time PCR using FRET technology was designed targeting the 28S rRNA gene of *Strongyloides* species. The assay was set up allowing the simultaneous detection of *S. stercoralis*, *S. fuelleborni*, *S. ratti*, *Strongyloides robustus*, *Strongyloides calloscinreus* and *Strongyloides procyonis*. To date, only *S. stercoralis* and *S. fuelleborni* are known to cause infection in humans but infection with other species might be possible. In addition, we aimed to provide a universal detection tool that can also be used in the *S. ratti* infection model.

Analytical sensitivity

Initial experiments were done with extracted DNA from *S. ratti* as well as *S. stercoralis* to optimize the assay with respect to reaction conditions. Following optimization, a plasmid,

pStrowild, was constructed and used to exactly determine the analytical sensitivity of the assay. Five copies per PCR of plasmid pStrowild were regularly detectable, indicating a high analytical sensitivity of the assay. A linear relationship between the real-time PCR crossing point and the \log_{10} input DNA copy number was observed over a wide range of concentrations (10^6 – 10^2 DNA copies per reaction).

An internal competitive control was incorporated into the assay. As single copies of pStroIC were detectable, this approach circumvents the need for multiplex PCRs, which in general can reduce sensitivity and reliability of assays. To exclude the possibility that the internal control affected the amplification efficiency of *Strongyloides* species detection, 20 copies of pStroIC per reaction were chosen as the working concentration for all further experiments. This working concentration did not reduce the overall sensitivity of the assay (data not shown) and has already been successfully incorporated into PCR assays using competitive internal controls (Panning *et al.*, 2007, 2008). In further experiments, the internal control was spiked into the lysis buffer and was co-purified with the sample. Cross-talk into the wild-type channel was not observed.

The 95% LOD was determined next. This common technical specification indicates the concentration down to which an assay will detect the analyte with at least 95% probability. In order to mimic the clinical situation, human stool specimens were spiked with plasmid pStrowild and served as reference material. Different concentrations of DNA were extracted as described above. Replicate testing of each analyte concentration yielded a 95% LOD of 18 DNA copies per PCR [95% confidence interval (CI) 14–32 DNA copies per PCR] equivalent to 1800 DNA copies per 200 mg stool (95% CI 1400–3200 DNA copies per 200 mg stool) (Fig. 2). The internal control yielded a valid signal in every reaction, indicating the robustness of the assay. As stool samples may contain a number of substances that are able to inhibit PCRs, an internal competitive control was introduced, which offered the opportunity to monitor for inhibitory activity throughout the whole extraction as well as amplification process. Furthermore, the use of a spin

column format to extract nucleic acids from stool samples has demonstrated its reliability in recent studies (Verweij *et al.*, 2000; Blessmann *et al.*, 2002). Next we determined the 95 % LOD using microscopically counted *S. ratti* larvae spiked into human stool suspensions as described above. Replicate testing yielded a 95 % LOD of one larva per PCR (95 % CI 0.8–1.5 larvae per PCR) equivalent to 100 larvae per 200 mg stool (95 % CI 80–150 larvae per 200 mg stool) (Fig. 2). This LOD is comparable to that of published real-time PCR assays (Panning *et al.*, 2008).

Specificity

To ensure a high specificity of the assay, FRET technology was applied, which relies on the concurrent binding of two probes in order to receive a fluorescence signal. This approach was chosen because of the sequence similarity within the group of nematodes. In contrast to a recent publication (Verweij *et al.*, 2009) in which one Taqman-minor groove binder probe for amplicon detection was utilized, FRET technology might further reduce non-specific binding. Specificity of this assay was determined by testing various DNA preparations derived from different helminth species, in particular nematodes, without yielding positive amplification results with material other than *Strongyloides* species. Cross-reactivity was also tested on a panel of common bacteria and viruses with negative results.

Additionally, 35 stool extracts from symptomatic patients without microscopic detection of parasites yielded negative results upon testing by the novel real-time PCR. To exclude false positivity, 23 DNA stool preparations from patients with other microscopically confirmed parasitic diseases were tested without yielding positive results with the *Strongyloides* species assay. This finding is in concordance with Verweij *et al.* (2009), indicating the high specificity of the novel assay. Due to a lack of DNA from *S. fuelleborni*, *S. robustus*, *S. callosinreus* and *S. procyonis*, we could not confirm the specificity of the assay for these pathogens. However, based on available sequence information, it is to be expected that these *Strongyloides* species will also be detectable with similar sensitivity (Fig. 1).

Clinical applicability

Clinical applicability of this assay could be demonstrated on five human stool samples with microscopic demonstration of *S. stercoralis* larvae. All specimens yielded a positive result upon use of the novel real-time PCR and subsequent sequencing of a fragment of the 28S rRNA gene confirmed the presence of *S. stercoralis* DNA. Recently, a real-time PCR has been evaluated on a panel of patients rendering it a useful tool for diagnostics (Verweij *et al.*, 2009). Compared to culture-based methods, it is suggested that real-time PCR can assist in the rapid diagnosis of strongyloidiasis especially when increasing numbers of patients have to be analysed (Blessmann *et al.*, 2002). However, it should be noted that the sample input volume

of different methods significantly influences diagnostic yield as already speculated (Verweij *et al.*, 2009). In this respect, larger studies are mandatory to compare classical and molecular methods in detail. In addition, as a future prospect in endemic countries, more inexpensive methods are desirable.

Application in an animal model

Finally, a well-established rat model for *S. ratti* was used to further test the applicability of the assay. Rats were infected subcutaneously with infectious stage 3 larvae (iL3) of *S. ratti*. Stool samples were analysed by microscopy as well as by real-time PCR from day 0 to day 40 post-infection (p.i.). Each sample was tested in duplicate. Real-time PCR yielded positive results from day 4 p.i. onwards (Table 1), whereas microscopy became positive for the detection of first-stage larvae on day 5 p.i. PCR remained positive until day 22 whereas microscopy allowed larval detection until day 18 only. Although real-time PCR was found to be more sensitive, further studies are needed to compare the outcome of microscopy and real-time PCR. The target region, the 28S rRNA sequence, exists in various copies. Even though the exact copy number is not known, the probability of detection should be increased compared to that of a single copy gene. The concept of targeting multicopy genes has already been successfully used for other pathogens (Panning *et al.*, 2008).

Conclusion

We demonstrated that this novel real-time PCR method can serve as a useful tool in both the clinical as well as a research setting. Analytical sensitivity and specificity are high and the assay meets today's standards of modern molecular diagnostic tools, i.e. an internal control system and quantification capabilities. It may thus assist decision making and therapy follow-up in the routine diagnostic laboratory as well as in research settings.

Table 1. Results of microscopy and real-time PCR within 23 days post-infection (p.i.) of Wistar rats with infective larvae

Each stool sample was analysed in duplicate. +, Positive result; –, negative result.

Result days p.i.	Real-time PCR	Microscopy
Day 0	–/–	–/–
Day 1	–/–	–/–
Day 2	–/–	–/–
Day 3	–/–	–/–
Day 4	+/+	–/–
Day 5	+/+	+/+
Days 6–18	+/+	+/+
Days 19–22	+/+	–/–
Day 23	–/–	–/–

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