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## Original Article

# Molecular Diagnosis of *Strongyloides stercoralis* Infection by PCR Detection of Specific DNA in Human Stool Samples

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## ABSTRACT

**Background:** Strongyloidiasis is mostly an asymptomatic infection and diagnosis of latent infections is difficult due to limitations of current parasitological and serological methods. This study was conducted to set up a PCR-based method for molecular diagnosis of *Strongyloides stercoralis* infection by detection of copro-DNA in stool samples.

**Methods:** A total of 782 fresh stool samples were collected and examined by agar plate culture. Among those sixteen stool samples, which confirmed to be infected with *S. stercoralis* were examined as positive control to set up each single and nested PCR, using two primer sets designing to amplify partial ribosomal DNA of *S. stercoralis* genome. Since, single PCR method yielded higher efficacy in detecting positive samples, in the second step, 30 stool samples, which found negative for *S. stercoralis* by agar plate culture of single stool sample, were examined by single PCR. Data analysis was performed using McNemar's  $\chi^2$  test, with consideration of a *P*-value of <0.05 as indication of significant difference.

**Results:** In amplification of DNA extracted from stool samples, single PCR detected *S. stercoralis* DNA target in all 16 positive samples, while nested PCR amplified DNA in only 75% of samples. In the second step, single PCR amplified *S. stercoralis* extracted DNA in 5 out of 30 samples which were negative by coproculture.

**Conclusion:** Single PCR method amplifying a short (100bp) target represented more efficacies for detection of *S. stercoralis* in faecal examination compared to agar plate culture and nested PCR, which amplified longer target.

**Keywords:** *Strongyloides stercoralis*, Diagnosis, Copro-DNA, PCR

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## Introduction

**S**trongyloidosis is an intestinal infection in humans caused by the nematode *Strongyloides stercoralis*, distributed in tropical and temperate areas (1, 2). In normal healthy individuals, the infection is usually asymptomatic, with low minimal and intermittent larval excretion. However, in some predisposing conditions like initiation of immunosuppressive therapy, hematologic malignancies, kidney transplant recipients and diabetics the disease may change to any forms of hyper infection or disseminated types of strongyloidosis (2-5).

Detecting latent cases of *S. stercoralis* decreases morbidity and mortality of the infection. The detection rate of conventional methods is low and repeated examinations of stool over a number of consecutive days is essential for diagnosis (6, 7). Several serodiagnostic tests with variable sensitivity and specificity have been studied for diagnosis of *S. stercoralis* (8-13). However, use of these methods has some limitations; source of antigen is necessary and if the test is positive, microscopic analysis is also necessary. These methods also do not show the level of larval excretion (5, 7).

In recent years, some PCR-based techniques have been developed and used for detection of different intestinal parasites in faecal samples (14-18). Evaluation and standardization of such techniques are necessary to overcome the limitations of the current diagnostic methods. Hence, the aim of the present study was to set up a PCR method for diagnosis of *S. stercoralis* infection by examination of stool samples.

## Materials and Methods

### *Samples collection*

Seven hundred and eighty two fresh stool specimens were collected from two endemic

provinces of strongyloidosis in Iran, including Mazandaran Province in north and Khuzestan Province in south-west of the country, and also from patients referred to the Helminthological Laboratory of School of Public Health, Tehran University of Medical Sciences, for parasitological examinations. Sixteen culture-positive stool samples were used as positive control for setting up the PCR. Thirty samples which found negative for *S. stercoralis* by agar plate culture of single stool sample were randomly selected for PCR test. Furthermore, 5 stool samples, reported negative for parasites by direct smear, formalin-ether concentration technique and agar plate copro-culture on three consecutive stool samples, were used as negative controls. For molecular examinations, all stool samples were preserved in 70% ethanol at room temperature.

### *Coprological examination*

Coprological examination for detecting *S. stercoralis* infected samples was conducted by copro-culture of single stool sample on agar plate medium as used by Arakaki et al. (19) and the plates were examined as explained by Kia et al. (20). A skillful parasitologist performed the morphological differentiation of the L3 larvae of *S. stercoralis* from other possible nematodes, especially *Rhabditis* spp. Filariform larvae of *S. stercoralis* were collected from positive agar plates by washing the surface of the agar plates with a phosphate buffer saline solution. The extracted DNA from filariform larvae was used as control DNA during molecular assays.

### *Extraction of genomic DNA*

About 3 g of each stool sample preserved in 70% ethanol alcohol was emulsified in 4% acetic acid. The suspension passed through two layers gauze into a tube, and after add-

ing 3ml ether, was shaken vigorously and centrifuged at 1000 rpm for 2 min. The pellet was washed twice with distilled water and then used for extraction of genomic DNA, using QIAamp® DNA stool MiniKit (QIAGEN, Hilden, Germany). In this way 1.4 ml of ASL buffer was added to the sample and put in 80°C water bath for 5 min. Later, the procedure continued according to the protocol for extraction of DNA from stool. The extracted DNA was finally eluted with 50µl AE buffer.

#### Single PCR

Forward (SSF: 5' ATC GTG TCG GTG GAT CAT TC 3') and reverse (SSR: 5' CTA TTA GCG CCA TTT GCA TTC 3') primer pair was designed using DNASIS software and based on alignment of rDNA sequences related to *S. stercoralis*, deposited in GenBank (Accession numbers: **EF653266**, **EF653265**, **EF653264**, **EF545004**) to amplify a 114bp target in rRNA gene. PCR reactions were performed using the following reaction mixture: 2X red PCR Mastermix (ROVALAB, Hauffstr, Germany), 25pmol of each primer, 1µl of template, and enough distilled water up to final volume of 25µl under following conditions: 1 cycle at 95°C for 5 min (time-delay), 30 cycle at 94°C for 30s (denaturation), 58°C for 45s (annealing) and 72°C for 45s (extension), followed by a final extension for 5 min.

The specificity of the primers was evaluated using DNA extracted from some gastrointestinal parasites including *Hymenolepis nana*, *Trichostrongylus colubriformis*, *Giardia lamblia*, *Entamoeba histolytica* and *Entamoeba coli* (3 samples of each), as well as using DNA extracted from *Candida albicans*, *Escherichia coli*, *Cytrobacter* spp., and distilled water as negative controls. The in silico specificity of primers for *S. stercoralis* in the NCBI BLAST was 100%.

#### Nested PCR

PCR reactions for both rounds were performed in 25µl volumes using 2X red PCR Mastermix (Ampliqon), 25pmol of each primer and 1µl of faecal DNA sample. For the primary amplification round, primers SSF0 (Forward: 5' ATC CTT CCA ATC GCT GTT GT 3') and SSR0 (Reverse: 5' TTT CGT GAT GGG CTA ATT CC 3') (21) were used to amplify a PCR product of 750bp containing ITS-1, 5.8s and ITS-2. For each set of PCR reactions, negative controls (distilled water and DNA extracted from negative stool samples) and positive controls, were included. The cycling conditions comprised an initial denaturation step at 95°C for 7 min, 30 cycles of denaturation at 94°C for 45s, annealing at 55°C for 90s, extension at 72°C for 90s, followed by a final extension at 72°C for 5 min.

Subsequently, 1µl of 1/10 diluted of the first round amplicon was subjected to a second amplification round, using primers SSFI (Forward: 5' GTA ACA AGG TTT TCG TAG GTG AA 3') and SSRI (Reverse: 5' ATT TAG TTT CTT TTC CTC CGC TT 3'). A product of 680bp was amplified under the following conditions: Initial denaturation at 94°C for 3 min, and 30 cycles of 94°C for 45s, 60°C for 45s and 72°C for 1 min, followed by a final extension for 5 min.

#### Electrophoresis

The products of single PCR and nested PCR were loaded on 2% and 1.5% TBE (Tris 0.09M – borate 0.09M – EDTA 0.02M) agarose gels (Bio life, Italina S.r.l, Italy), respectively. The gels contained 0.5µg/ml ethidium bromide (Roche, Germany) for staining. Electrophoresis carried out 1 hour at 80V. Ultraviolet light was used to visualize the stained DNA.

#### Data analysis

In order to determine whether the observed difference between the results of the tests is

statistically significant McNemar's  $\chi^2$  test was employed, with consideration of a *P*-value of <0.05 as indication of significant difference.

## Results

### Agar plate culture

In examination of 782 stool samples by agar plate culture 16 cases were infected with *S. stercoralis* which all were examined as positive control to set up each single and nested PCR. During microscopic examination of the surface of agar plates of infected cases either homogonic or heterogonic life cycle of *S. stercoralis* (Fig.1) could be observed.

### Single PCR

A PCR product of 114bp was amplified with designed primers using genomic DNA isolated from precipitant of *S. stercoralis* infected faecal samples (Fig. 2). All 16 (100%) previously confirmed *S. stercoralis* infected faecal samples were positive by single PCR assay. Among 30 randomly selected stool samples which found negative for *S. stercoralis* by agar plate culture of single stool sample, for 5 samples the PCR amplification became positive with the expected band size. No amplification was detected for any above mentioned negative control samples.

Overall, in examination by single PCR, 21 out of 46 stool samples were found positive

for *S. stercoralis* while by agar plate culture for the same stool samples the detected positive cases was lower (Table 1).

### Nested PCR

Partial coding and non-coding spacer regions of rDNA were amplified using genomic DNA extracted from faecal samples. As for single PCR amplification, extracted DNA from stool precipitation was used in nested PCR assay. All the PCR amplicons produced at the second PCR round represented band of approximately 680bp. Among 16 previously confirmed *S. stercoralis* infected faecal samples, only 12 samples (75%) were found positive in the two-step nested PCR rounds. No amplification was detected for any negative control samples.

As the efficacy of nested PCR for the detection of *S. stercoralis* confirmed stool samples was lower than single PCR ( $\chi^2=4$ ), further examinations were performed only by single PCR assay (as described above).

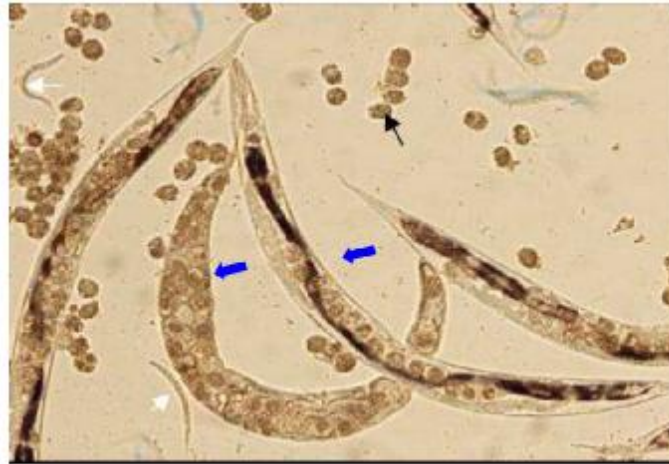
### Statistical analysis

Statistical analysis using McNemar's  $\chi^2$  test revealed that the difference between the result of single PCR and agar plate culture for the detection of *S. stercoralis* infected faecal samples is significant ( $\chi^2=5$ ) and single PCR was able to detect more infected cases (Table 1).

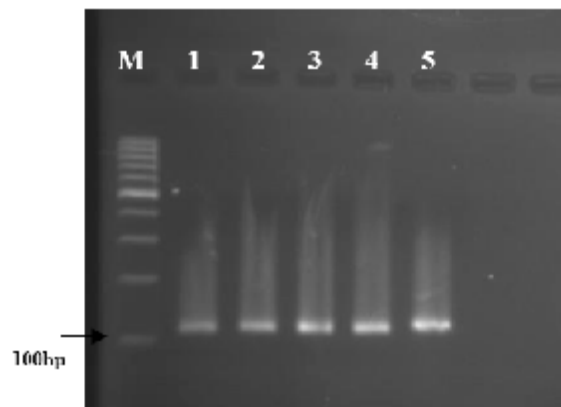
**Table 1:** Comparison of the results of single PCR and agar plate culture examinations for detection of *Strongyloides stercoralis* infection in single stool samples

	Agar plate result	Single PCR		Total
		+	-	
Agar plate coproculture	+	16	0	16
	-	5	25	30
Total		21	25	46

$P < 0.05$ ;  $\chi^2 = 5$



**Fig. 1:** Surface of an agar plate culture showing heterogonic life cycle of *Strongyloides stercoralis* (black arrow: egg, white arrow: first- stage larva, center arrows: free-living female)



**Fig.2:** Agarose-gel electrophoresis of the single PCR products amplified by primers targeted the partial rDNA of genomic DNA extracted from precipitated stool samples infected with *Strongyloides stercoralis*

Lanes 1-5: PCR products of 5 stool samples infected with *S. stercoralis*  
M: 100bp DNA marker ladder

## Discussion

In the majority of uncomplicated cases of strongyloidiasis, the intestinal worm load is very low and the output of larvae is minimal (22). This issue can encounter the immunocompromised infected patients to endangered conditions, leading the infection to uncontrolled disseminated forms. Therefore,

development of highly sensitive diagnostic tests to detect light cases of strongyloidiasis is crucial to prevent potentially fatal infections. Among different parasitological methods, agar plate culture of stool sample has been reported as method that is more sensitive to detect *S. stercoralis* infections (20,

23, 24); however, this method requires multiple fresh stool sampling and experienced microscopist. Accordingly, development and evaluation of PCR based methods, as suitable alternative methods in order to increase detection rates, are needed. In several studies, PCR has been stated as highly sensitive method for detection of both protozoan (14) and helminthic (15-17) infections in faecal samples.

A multiplex real-time PCR, focusing on four target parasites including *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium* and *S. stercoralis* in returning travelers showed to be highly sensitive and specific technique compared with the routine approach of microscopy and antigen-based methods (18). However, PCR inhibitors are relatively common in stool samples and they can strongly influence the results of PCR assays (25). This inhibiting effect is more noticeable in samples with lower parasite DNA (15). Therefore, in the current study in order to increase the amount of faeces used for PCR assay, the stool sample was concentrated by acid-ether prior to DNA extraction. Then, nested PCR and single PCR were established for the detection of DNA of *S. stercoralis* in the faecal samples. Both assays were highly specific, and no amplification was detected for any negative control samples used. However, the sensitivity of single PCR was higher than nested PCR. In nested PCR only in 75% of infected samples (12 out of 16) *S. stercoralis* DNA were amplified; while in single PCR all positive samples were detected. The false negative results of nested PCR might be due to the size of amplified fragment which was very small in single PCR. In the study of Verweij et al. *S. stercoralis* real-time PCR achieved 100% specificity and high sensitivity, with a two fold increase in the detection rates compared with Baermann sedimentation method (17).

In the present study, single PCR assay not only detected all infected samples that were found positive by agar plate culture of single stool sample but also detected some infected cases that agar plate culture of single stool sample was not able to recognize them. Therefore, single PCR was more efficient in detecting *S. stercoralis* infected faecal samples and this difference between efficacy of these two methods was statistically significant ( $P < 0.05$ ). Therefore, in cases that the worm burden is too low or larvae are not alive to be detected on agar plate culture, the application of PCR will be useful.

In conclusion, the result of the current study shows that performing single PCR, complemented with concentration of larvae in stool by acid-ether technique before DNA extraction, provides highly specific and sensitive molecular method for diagnosis of *S. stercoralis* genome in human faeces. Further studies to find the effect of multiple stool sampling on detection rate of *S. stercoralis* infections by PCR based methods is recommended. In addition, the development of multiplex PCR for several target parasites can be applied for detection of infections in immunocompromised people who are at risk of disseminated strongyloidosis and other opportunistic infections.

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