

## A DNA-Based Identification of *Strongyloides stercoralis* Isolates from Iran

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### Abstract

**Background:** *Strongyloides stercoralis*, the etiological agent of strongyloidiasis, is one of the most common parasitic nematode with the unique ability to complete its life cycle and proliferate within the host. Although it is an endemic parasite in Iran, no molecular characterization is available on isolates originated from the country. Therefore, this study was conducted for molecular identification of human *Strongyloides* isolates in the three most prevalent provinces.

**Methods:** After microscopical examination and agar plate culture of nearly 1500 stool samples, 20 isolates of *S. stercoralis* filariform larvae were recovered from (Gilan and Mazandaran in north and Khouzestan in south) of Iran. The genomic DNA was extracted from all these samples and two primer sets were selected for amplification. ITS1 region of the rDNA gene was amplified by a nested Polymerase Chain Reaction (nested-PCR). The PCR products were sequenced and analyzed in comparison with the sequences deposited in GenBank.

**Results:** DNA sequence analysis of ITS1 region showed that all the 20 isolates were *S. stercoralis*. There was slight variation in the ITS1 region among the isolates.

**Conclusion:** ITS1 sequencing seems to be a valid target for molecular identification of *S. stercoralis*.

**Keywords:** *Strongyloides stercoralis*, rDNA, Iran

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### Introduction

*Strongyloides stercoralis* is an intestinal nematode of humans estimated affecting tens of millions of persons worldwide (1). This parasite is endemic in tropical and subtropical areas. *S. stercoralis* can transform into a fulminant fatal illness in immunocompromised patients. With the increased mortality and morbidity associated with *S. stercoralis*, focusing on identification, screening, and treating those at risk is warranted (1-3). Traditional methods for diagnosis of the infection are based on the cultivation of larvae and microscopic morphology for differentiation of third-stage larvae (L3) (4). However, this approach is tedious and not always reliable due to minor morphological characteristics.

Recently, molecular biological assays have been developed to detect specific DNA of gastrointestinal nematodes (5, 6). Ribosomal DNA se-

quences, including the Internal Transcribed Spacer (ITS) regions, have been used in the studies to design radioactive DNA probes or specific PCR primers. A few studies on platyhelminthes (7-9) have indicated the potential of the sequences of different parts of rDNA for their specific identification. The ITS region was particularly well suited as a target because it is relatively short and repetitive in nature, making any diagnostic PCR assay sensitive (10).

*S. stercoralis* has long been an endemic nematode in Iran, and as like as other endemic areas in the world, it is primarily occurs in tropical and subtropical regions (11). Although it is a health threatening parasite and to date several cases of strongyloidiasis hyper infection syndrome have been reported in the country (12), however, there is no report about the molecular aspects of this parasite.

The present study was undertaken to perform identification of *S. stercoralis* isolates from endemic areas of Iran, based on the ITS1 region of the rDNA gene.

## Materials and Methods

**Parasite isolation:** Overall, about 1500 stool samples were collected from residents of the most prevalent provinces including Mazandaran and Gilan provinces in north and Khouzestan Province in south of Iran, and examined for *S. stercoralis* by formalin ether concentration method and agar plate culture (13). For agar plate culture, 3 g of every fecal sample was placed in the center of nutrient agar plate. The plates were then incubated for 5 d at room temperature (26-30 °C). From following day onwards the plates were examined under the microscope for the presence of moving larvae or free-living adults or their tracks on the surface of the agar for five consecutive days. All microscopically positive samples were further processed by washing the surface of the agar with a PBS (Phosphate buffer saline) solution and/or by using Baermann apparatus to collect filariform larvae of *S. stercoralis* (14). Considering morphological characters of the L3 larvae, differential diagnosis from other possible nematodes, especially *Rhabditis* spp. and *Trichostrongylus* spp., was fulfilled. The samples were preserved in 75% ethanol solution and kept at ambient temperature for subsequent molecular processing.

**DNA extraction** Approximately 300 µl final suspension of each sample preserved in 70% ethanol was suspended in 250 µl of lysis buffer (200 mM Tris-HCl, 100mM NaCl, 30 mM EDTA), 25 µl of 10% SDS solution and 10 µl of 10 mg/ml proteinase K solution. Then, the suspension was mixed thoroughly, and incubated at 56 °C for 90 min. After centrifugation at 10000 rpm for 10 min, the supernatant was extracted once with phenol-chloroform-isoamyl alcohol and subsequently by chloroform-isoamyl alcohol. Then the supernatant, was recovered and transferred to a fresh 1.5 ml centrifuge tube, adding 0.1 volume of 3 M sodium acetate solution (pH=5.2) and an

equal volume of 2-propanol, followed by incubation at -20 °C for 10 min. After centrifugation at 12000 rpm for 15 min, the supernatant was discharged and the sediment was washed with chilled ethanol (70%), air-dried and resuspended in 50 µl distilled water. The DNA extracts were then stored at -20 °C until PCR amplification. In some cases, QIA amp DNA minikit (Qiagene Company, Germany) was used for purification of extracted DNA.

**PCR amplification** ITS1 region of the rDNA gene was amplified by a nested-polymerase chain reaction (nested-PCR). Primers for the ITS1 region were designed by using DNASIS software (Hitachi, Japan). Primer selection was based on alignment of 18S and 28S ribosomal DNA related to various species of *Strongyloides* deposited in GenBank (Accession numbers: SSU39489, AF279916, M84229, AJ417023). For the primary amplification, a PCR product of 750-bp was amplified using the forward (SS-FO: 5'-ATC CTT CCA ATC GCT GTT GT-3') and reverse (SS-RO: 5'-TTT CGT GAT GGG CTA ATT CC-3') primers. The PCR reaction (total volume of 50 µl) contained 5 µl of 10×PCR buffer, 2.5 µl MgCl<sub>2</sub>, each deoxynucleoside triphosphate at a concentration of 200 µM, each primer at a concentration of 100 nM, 2.5 U of *Taq* polymerase, and 1 µl of DNA template. The amplification reactions of 35 cycles were initiated by denaturation of the DNA at 94 °C for 5 min, denaturation at 94 °C for 45s, annealing of the primer at 58 °C for 60 s, and extension at 72 °C for 1 min, with an additional 5 min extension at 72 °C. For the secondary PCR, a product of 680 bp was amplified using 1 µl of primary PCR product and forward (SS-FI: 5'-GTA ACA AGG TTT TCG TAG GTG A-3') and reverse (SS-RI: 5'-ATT TAG TTT CTT TTC CTC CGC TT-3') primers. The second PCR reaction (nested-PCR) consisted of an initial denaturation at 94 °C for 2 min, and 30 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min followed by a final extension for 5 min. Negative (water instead of DNA template) control, was included in each amplification run. Amplified prod-

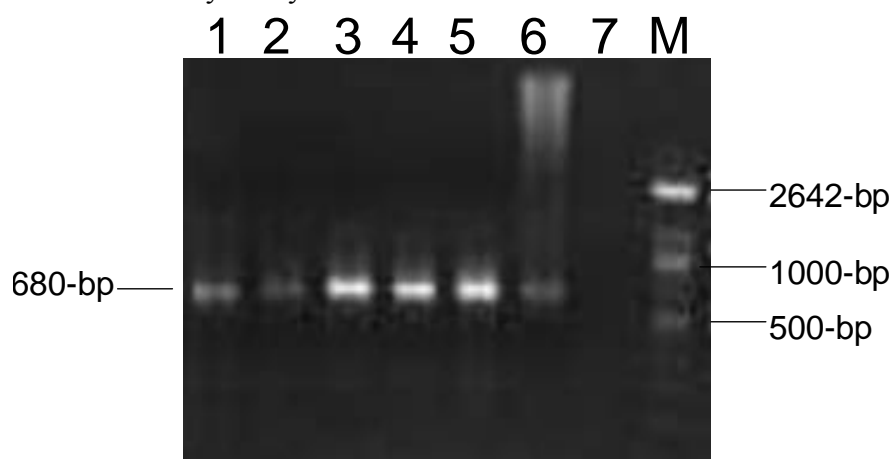
ucts were visualized by 1.3% (W/V) agarose gel electrophoresis in TBE buffer (0.09M Tris, 0.09M Boric acid and 0.02M EDTA, pH: 8.3), stained with ethidium bromide (0.5 microgram per ml) and photographed.

**Sequencing:** All secondary PCR products were sequenced by SEQLAB company (Germany). The nucleotide sequences were aligned with those from the GenBank database, using Blast ([www.ncbi.nlm.nih.gov/Blast](http://www.ncbi.nlm.nih.gov/Blast)) software.

## Results

Among nearly 1500 stool samples, 30 cases were found infected with *S. stercoralis* by agar plate culture. However, for 20 isolates extraction of genomic DNA was successfully achieved. All these 20 isolates were then analyzed by molecu-

lar methods. The isolates were characterized for the ITS1 region of the rDNA gene by nested-PCR technique and sequencing. An initial 750-bp fragment of genomic DNA was amplified using SS-FO and SS-RO primers, followed by nested PCR using SS-FI and SS-RI primers to amplify an internal fragment of 680-bp (Fig. 1). The PCR products were specific for *Strongyloides*, and no bands were observed in negative control (water instead of DNA template). DNA sequence analysis of ITS1 region showed that all the 20 isolates were *S. stercoralis*. The nucleotide sequences of the ITS1 region related to one example of the *S. stercoralis* isolates are presented in Fig. 2. There was slight difference in the nucleotide sequences of the samples.



**Fig. 1:** Molecular diagnosis of *Strongyloides stercoralis* by a nested PCR based on rDNA gene. Line M: molecular weight marker XIV; line 7: negative control; line1-6: patients isolates

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AAGACATTATGATAAAAATATAATTATTTTATAATATATAAAAATATTTTATAATAAACACAT
AAGATGGTACGTAAAAAAAATTTAAAAAAATAAATTTTATTATTATTTTAAATAAACG
TTTAAATAGATATTTTAATTAATATCAGCCATCAAATATTATTTTAATATATAATTTTTTTT
ATTAAATAAAAATAAAAATTTTTTTATAAAAATCGTGTCGGTGGATCATTCGGTTCATAGGT
CGATGAAGAGCGCAGCTAAATGCGATAAGTAAGGTGAATTGCTAAGCAGAGCCTTAAATT
CTTGAATGCAAATGGCGCTAATAGTATTTTATACTATTGGCATATATAATTGAGGGTGATA
ACAAGTAGTATTGTATATTATTATTTTATATTAATAAATAAATAATATATTAATAAAAATAT
TTTTAATTTTTTAAATAAAAGTATTTTTATTGTATATTTTTTTTTTTTATTATATAAAAAAAA
TAAAATATCAGGTTGAAATATATNAAATTTTTTTNAAAAAAAAAAAAATCATTAAAAAT
TTTTTAAATTTTTTT
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**Fig. 2:** An example of nucleotid sequences at the ITS1 region related to one of the *S. stercoralis* from Khuzestan isolates

## Discussion

The potential implications for studying the epidemiology and population biology of parasites offered by PCR assays seem highly valuable (15). DNA-based techniques have provided useful alternatives for the accurate identification of *Strongyloides* species, irrespective of developmental stage. For instance, PCR-based restriction fragment length polymorphism (PCR-RFLP) was used to differentiate several species of *Strongyloides*, and it was found that the four human isolates from different geographic areas of the world had identical patterns with the nine restriction enzymes used. In addition, the differentiation between the dog isolates and human isolates of *S. stercoralis* could be possible (16). The evaluations for 100 species of nematodes representing the orders Strongylida and Ascaridida revealed that the magnitude of within-species sequence variability (irrespective of developmental stage, gender and geographical origin) was significantly lower than the differences between species (17- 19). This finding indicated the usefulness of the ITS sequences for the specific identification of parasitic nematodes, which had important implications, both fundamental and applied.

In this study, a range of *S. stercoralis* isolates, originally retrieved from human, were characterized by amplification of the ITS1 region of the rDNA gene. Among 20 isolates characterized, 14 isolates showed homogonic development and the others heterogonic development in nutrient plate cultures. However, by the nested-PCR, all the isolates showed an identical pattern, with a visible fragment of 680-bp in size. For all 20 isolates nucleotide sequencing were achieved, and comparison with other available sequences in GenBank was performed, using blast program. According to the results, the identity of all the 20 isolates confirmed as *S. stercoralis*. It is postulated that using PCR with specific primer could be useful for molecular analysis, diagnosis and epidemiological studies.

We are extending the results of the present study by testing larger numbers of specimens from hu-

mans with strongyloidiasis, and applying methods with increased discriminatory powers.

To sum up, in the present study a sensitive method, that uses DNA extracted from human fecal samples, is reported for the identification of *S. stercoralis*. The PCR method is specific, robust and reproducible. The authors believe that this approach, together with the development of more discriminatory typing methods, will vastly increase the understanding of the epidemiology of strongyloidiasis. Methods with improved sensitivities, such as the one described here, will also be valuable in the detection and characterization of *Strongyloides* in non-human hosts.

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## References

1. Keiser PB, Nutman TB (2004). *Strongyloides stercoralis* in the immunocompromised population. *Clin Microbiol Rev*, 17: 208-17.
2. Kroshnam S, Barger G, Chandrasekar P (2006). Recurrent *Strongyloides stercoralis* hyperinfection syndrome. *Infect Dis Clin Prac*, 14: 240-43.
3. Rahim S, Drabu Y, Jarvis K, Melville D (2005). Strongyloidiasis: a mistaken diagnosis and a fatal outcome in a patient with diarrhea. *Trans R Soc Trop Med Hyg*, 99: 215-17.
4. Burger HJ, Stoye M (1968). *Parasitologische Diagnostik (Teil II)*. Therapogen Praxisdienst, Therapogen, Munich, pp. 1-24.

5. Schnider T, Heise M, Epe C (1999). Genus-specific PCR for the differentiation of eggs and larvae from gastrointestinal nematodes of ruminants. *Parasitol Res*, 85: 895-98.
6. Samson-Himmelstjerna GV, Harder A, Schnider T (2002). Quantitative analysis of ITS2 sequences in *Trichostrongyle* parasites. *Inter J Parasitol*, 32: 1529-35.
7. Luton K, Walker D, Blair D (1992). Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). *Mol Biochem Parasitol*, 56: 323-28.
8. Adlard RD, Barker SC, Blair D, Cribb T (1993). Comparison of the second internal transcribed spacer (ribosomal DNA) from populations and species of Fasciolidae (Digenea). *Inter J Parasitol*, 23: 423-25.
9. Hancock K, Broughel DE, Moura INS, Khan A, Pieniazek NJ, Gonzalez AE (2001). Sequence variation in the cytochrome oxidase I, internal transcribed spacer 1, and Ts14 diagnostic antigen sequences of *Taenia solium* isolates from South and central America, India, and Asia. *Inter J Parasitol*, 31: 1601-7.
10. Gasser RB (2001). Molecular taxonomic, diagnostic and genetic studies of parasitic helminthes. *Inter J Parasitol*, 31: 860-64.
11. Berk SL, Verghese S, Alvarez K, Hall K, Smith B (1987). Clinical and epidemiologic features of strongyloidiasis. A prospective study in rural Tennessee. *Arch Intern Med*, 147: 1257-61.
12. Kia EB, Meamar AR, Mahmoudi M, Mohraz M, et al. (2004). *Strongyloides stercoralis* hyperinfection syndrome in HIV+/AIDS patients and in immunocompetent hosts in Iran. Proceedings of IX European Multicolloquium of Parasitology, 18-23 July. 2004, Valencia, Spain.
13. Arakaki T, Iwanaga M, Kinjo F, Saito A, Asato R, Ikeshiro T (1990). Efficacy of agar plate culture in detection of *Strongyloides stercoralis* infection. *J Parasitol*, 76: 425-8.
14. Markell EK (2006). Examinations of stool specimens. In: *Medical Parasitology*. Ed, EK Markell and M Voge. Saunders 9<sup>th</sup> ed. Missouri, p.407-8.
15. Gasser RB, Newtuo SE (2000). Genomic and genetic research on bursate nematodes. Significance, implications and prospects. *Inter J Parasitol*, 30: 509-34.
16. Ramachandram S, Gam AA, Neva FA (1997). Molecular differences between several of *Strongyloides* and comparison of selected isolates of *S. stercoralis* using a polymerase chain reaction linked restriction fragment length polymorphism approach. *Am J Trop Med Hyg*, 56(1): 61-3.
17. Hoste H, Chilton NB, Gasser RB, Beveridge I (1995). Differences in the second internal transcribed spacer (ribosomal DNA) between five species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *Inter J Parasitol*, 25: 75-80.
18. Campbell AJD, Gasser RB, Chilton NB (1995). Differences in a ribosomal sequence of *Strongylus* species allows identification of single eggs. *Inter J Parasitol*, 25: 359-65.
19. Hung GC, Chilton NB, Beveridge I, Gasser RB (2000). Molecular systematic framework for equine *Strongylus*. *Inter J Parasitol*, 30: 95-103.