Original Article

Molecular Characterization of *Strongyloides Stercoralis* in Mazandaran Province, North of Iran

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ABSTRACT

Strongyloides stercoralis is a parasitic nematode that lives in the mucosa of the small intestine and causes strongyloidiasis in humans. Mazandaran is among the endemic areas of this parasite in Iran. For detecting *S. stercoralis* larvae in stool samples, various techniques, such as PCR technique have been used. The present study was conducted to determine the molecular characteristics of *S. stercoralis* collected from residents of Mazandaran, Northern Iran. From April to September 2017, a number of 2,195 samples of human feces were collected from different regions of Mazandaran province. First, all stool samples were tested using the formalin-ether method. Then, *S. stercoralis*-positive stool samples and 300 random samples were selected for molecular study. A set of primer pairs for conventional PCR was used in a PCR reaction to amplify the mitochondrial cytochrome c oxidase subunit 1 (*Cox1*) gene. To confirm the results of PCR, positive samples were sent for sequencing. The sequence was compared with reference sequences from GenBank. Phylogenetic relationships of the *Cox1* gene of *S. stercoralis* inferred by the maximum likelihood algorithm. According to our results, in the stool test with the formal ether method, 21 (0.95%) stool samples were found to be positive for *S. stercoralis*, and 162 (38.7%) samples were positive for other parasites. All 21 positive samples were confirmed as *S. stercoralis* by PCR method. The sequence of the samples overlapped 99% with *S. stercoralis* in the Genbank. Our results showed that conventional PCR could detect all the microscopically positive samples.

Keywords: *Cox*1 gene, Iran, Molecular characterization, *Strongyloides stercoralis*

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1. Introduction

Strongyloides stercoralis, a parasitic nematode in humans, lives inside the mucosa of the small intestine and causes severe clinical manifestations of strongyloidiasis (1). Most infected individuals are asymptomatic, while some patients have a variety of cutaneous, gastrointestinal, or pulmonary symptoms (2). Clinical symptoms of strongyloidiasis include acute, chronic, and disseminated infections (3). The most severe complications of *S. stercoralis* infection occur in patients with immune deficiency and, as a result, infect and spread the larva to multiple organs, including the brain (4). This parasite has infected 100-200 million people in 70 countries (5). So far, the prevalence has been reported between 4% and 50% of several regions worldwide (6). A total of 347 deaths due to strongyloidiasis were reported in the United States from 1991-2006 (7). The definitive diagnosis of strongyloidiasis is usually based on observation of the larvae in the stool exam (8). However, in most uncomplicated cases, the amount of parasite in the intestine is very low (9). For detecting *S. stercoralis* larvae in stool samples, various techniques have been used. These methods include Baermann concentration, Harada-Mori culture, agar plate culture (APC), and Ethyl acetate formalin concentration (10). So far, different immunological diagnostic methods have been used with variable specificities and sensitivities. Although in endemic areas, serologic responses are well-established after treatment, they are not particularly specific due to the interaction with other parasites, including *Filariasis*, *Ascariasis*, and *Schistosomiasis* (11). The PCR techniques have progressed greatly and have been used to detect various intestinal parasites in fecal specimens (12). Recently, the detection of parasite DNA in fecal specimens using Real-Time PCR has proven that this method is specific and sensitive for the detection of *S. stercoralis* infections (13). Assessing such techniques is critical to overcome the limitations of current diagnostic methods. Strongyloidiasis is still endemic in some Iranian provinces, including Mazandaran. In 2007, the infection rate in the Mazandaran province was 4.9% (14). In a study conducted in Gilan province, 42% of the people with eosinophilia were reported positive for *S. stercoralis* infection (15). The present study was conducted to determine the molecular characteristics of *S. stercoralis* collected from residents of Mazandaran, the endemic region of the parasite in Iran.

2. Materials and Methods

2.1. Sample collection

From April 2017 to September 2017, a total of 2,195 human stool samples were collected from various regions of Mazandaran province, North of Iran. In this survey, individuals profile was as follows: 1,170 (53.3%) women and 1,025 (46.7%) men, 763 (34.8%) living in urban and 1,432 (65.2%) in rural areas, hospitalized people were 680 (31%), and outpatients referred to the hospital were 1,515 (69%).

2.2. Microscopic study

All stool samples were analyzed using the formalin ether method (16). After performing parasitic experiments, the obtained larvae were washed with distilled water several times and stored in 70% ethanol at 4°C for molecular analysis. The larvae of *S. stercoralis* were detected based on morphological characteristics with optical microscope and classification keys of nematodes (17).

2.3. DNA extraction

The positive fecal samples of *S. stercoralis* and 300 random samples were selected for DNA extraction. About 5 g of each fecal sample stored in 70% ethanol was dissolved in 4% acetic acid, and the suspensions were transferred to a tube through two layers of sieve, and then 3 ml of ether was added to it. Then, the tube was shaken slowly and centrifuged at 1500 g for 2 min. The pellet was washed three to four times with distilled water. The DNA of stool specimens was extracted by DNA purification kit (YTA Genomic DNA Extraction Mini kit for Tissue). In summary, the first 200ul of buffer TG1 and proteinase k was added to the samples. Then, incubated at 60°C for overnight. This method is carried out according to the manufacturer's instructions. Finally, the extracted DNA was eluted with 50µl Elution Buffer and was kept at -20°C for molecular study.

2.4. Conventional PCR

A set of primer pairs was used for conventional PCR to activate the amplification of the 509-bp target in a PCR reaction in the mitochondrial cytochrome c oxidase subunit 1 (*Cox*1) gene. For amplification of *Cox* 1 of *S. stercoralis*, primers *Cox*F (5′-TGG TTT GGG TAC TAG TTG-3′) and *Cox*R(5′-GATGAG CTC AAA CTA CAC A-3′) were used (18). The PCR reaction was done using the following reaction mixtures: 10 uL Taq DNA Polymerase 2X RED PCR Master Mix (Ampliqon, 2mM MgCl2), 10 pmol/µL of each primer, 250ng/µL of DNA template, and distilled water up to a final volume of 20 µL. The reaction was done for 35 cycles: (94°C for 30s (denaturation), 55°C for 30s (annealing), and 72°C for 30s (extension)), with an initial denaturation (1cycle at 94°C for 4 min) and a final extension (1cycle at 72°C for 4 min). To confirm the optimization of the reaction process, the extracted DNA from larvae of the filariform was used as a positive control, and three samples that were negative by the formalin ether method were used as a negative control. A 5 uL of PCR product was electrophoresed on a 1% agarose gel in Tris-Acetic acid EDTA (TAE) buffer, and the band was visualized using an ultraviolet

illuminator. To confirm the results of PCR, four positive products randomly were sent for two-way sequencing and sequenced by the ABI3730XL sequence analyzer (Macrogen, Korea). Sequences obtained edited and aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and compared with reference sequences from GenBank. Then, the phylogenetic tree was built for *S. stercoralis* with the maximum likelihood algorithm using molecular evolutionary genetics analysis software (MEGA), including sequences of demonstrator species of *Strongyloides* infecting humans from the GenBank.

2.5. Data analysis

All the collected data was analyzed using SPSS (version 18.0) to determine the significant statistical difference between observations using the χ^2 test.

3. Results

3.1. Microscopic study

A total of 2,195 stool specimens were examined in this study using optical microscopy, and 183 of them were found to be infected with different parasites. Twenty-one (0.95%) stool samples were found to be positive for *S. stercoralis*, and 162 (7.38%) samples were positive for other parasites (Table 1). Among these parasites, *Entamoeba histolytica/dispar*, *Giardia lamblia*, and *S. stercoralis* were the most frequent. Among the patients who had *S. stercoralis*, 15 (71.43%) people were male, 6 (28.57%) people were women, 3 (14.29%) patients were living in urban, 18 (85.71%) patients were from rural areas, hospitalized people were 6 (28.58%), and outpatients referred to the hospital were 15 (71.42%).

Table 1. The number and percentage of positive samples of *S. stercoralis* and other gastrointestinal infections based on microscopic detection in Mazandaran Province, North of Iran

Parasites	Microscopic	
	No. of positive	% of positive
	Helminths	
Strongyloides stercoralis	21	0.95
Hookworm	11	0.50
Trichiura Trichuris	4	0.18
Hymenolepis nana	4	0.18
Enterobius vermicularis	3	0.13
Taenia	1	0.04
	Protozoa	
Entamoeba histolytica/dispar	47	2.14
Entamoeba coli	30	1.36
Endolimax nana	25	1.13
Giardia lamblia	22	1.00
Iodamoeba butschlii	9	0.41
Blastocystis hominis	4	0.18
Dientamoeba fragilis	2	0.09
Total	183	8.33

3.2. Molecular analysis

The PCR product showed a band of~ 500bp in gel electrophorese (Figure 1). All 21 samples that confirmed *S. stercoralis* in fecal samples were found to be positive by PCR method. In addition, among 300 stool samples that were randomly selected, which were negative for *S. stercoralis* by formalin ether method, rechecked using the molecular method, and all of them were found to be negative.

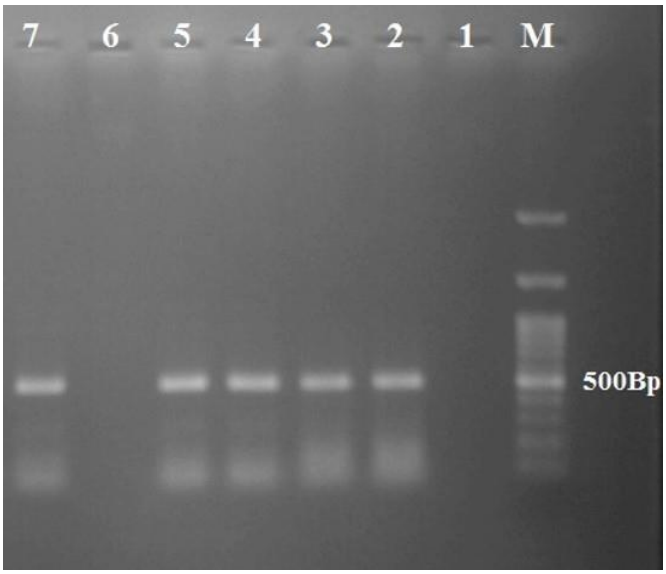

Figure 1. PCR product of *Cox 1* gene of *Strongyloides stercoralis* on 1.5 % agarose gel. M: 100bp DNA marker. Lane 1: Negative control. Lane 2: Positive control. Lanes 3-7: stool samples.

The sequence of the samples had 99% overlap with *S. stercoralis* in GenBank. The sequences isolated in this study are registered in the GenBank and are available with an Accession number of MH921561-64.

Phylogenetic relationships of the mitochondrial cytochrome c oxidase subunit 1 (*Cox1*) gene of *S. stercoralis* inferred by the maximum likelihood algorithm, are shown in Figure 2.

4. Discussion

Strongyloidiasis is very important in patients with immune deficiency, which causes hyperinfections and disseminated syndromes and can be fatal if not well treated (9, 10). This parasite is rife in the tropical and subtropical regions of the world, and about 100 million people in 70 countries have been infected with this parasite (19).

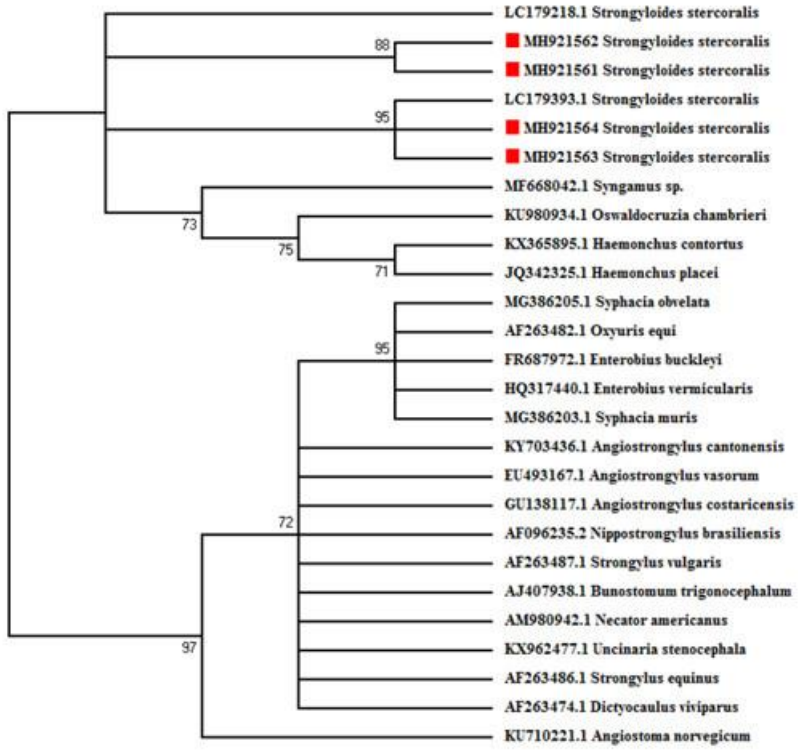

Figure 2. Phylogenetic relationships of the mitochondrial cytochrome c oxidase subunit 1 (*Cox*1) gene of *S. stercoralis* inferred by maximum likelihood algorithm, in which genotypes of this study were registered with MH921561-64.

In Iran, in the Northern and Southern coastal provinces where there is sufficient moisture, this parasite is reported endemic (20). During the last decades, due to improvements in the level of health and increasing public awareness, the prevalence of soil-transmitted parasites has declined substantially in the country. However, due to the fact that *S. stercoralis* causes autoinfection in the host body, it is still prevalent in the endemic areas. The sensitivity and specificity of various methods for the diagnosis of *S. stercoralis* infection have been reported differently in various studies. For this reason, the actual prevalence of the methods is often underestimated (14). The APC in stool specimens is among the best methods for diagnosing *S. stercoralis* (13). However, this procedure requires multiple samples of fresh stool and an experienced person for diagnosis. Therefore, PCR-dependent methods can be used as an appropriate alternative method for detecting *S. stercoralis* parasites. Various studies have been performed based on the PCR technique, and each has shown different results in detecting *S. stercoralis* DNA in stool samples (11). In this study, a molecular method was selected for the validation and verification of *S. stercoralis* specimens, and contributed to insight into its prevalence in the studied specimens. The prevalence (0.95%) for *S. stercoralis* in this study is almost similar in comparison to other studies conducted in Iran. Although, there are studies that are not consistent with our research. In 2016, among people who were mentally impaired in Gilan province, 1.2% of them were found infected with this parasite (21). Ghasemikhah et al. (2017) studied 1,800 patients in Tabriz and reported the prevalence of S. *stercoralis* to be 0.3%, which is lower than our study (22). In other studies conducted in Iran, the rate of infection of this parasite varies with respect to the target population and diagnostic methods. In a study conducted by Sharifdini et al. (2018), based on nested PCR, 9.7% of people in the Northern province of Khuzestan were infected with *S. stercoralis* (23). Kia et al. (2007), with parasitological methods, revealed that 4.9% of rural residents in Mazandaran province were infected with S. *stercoralis* (13). Among those who were in mentally retarded institutions in southern Iran in 2012, the prevalence of *S. stercoralis* was reported at 3.17%, using the formalin ether concentration method (24). In addition, the rate of this parasite was reported 2.1% in the people of the rehabilitation center of Mazandaran province in 2015 (25). The present study showed that males are more infected with *S. stercoralis* than women (71.5% versus 28.5%; $P<0.05$). Several studies have shown that men are more infected than women (19). This can be due to outdoor activities and the work of man in agricultural and horticultural land. The selection of an appropriate method

for detecting strongyloidiasis to determine its prevalence can help us in the control and prevention program. The PCR technique is a highly sensitive and specific method for detecting protozoans and parasitic infections in fecal specimens (11). The results of this study showed that Single-PCR could detect all specimens that microscopically were positive. In addition, a suitable and cost-effective method can be used to detect *S. stercoralis*. The study of Moghaddassani et al. (2011) showed that Single-PCR was more effective than Nested-PCR in detecting *S. stercoralis* in stool samples (26). The differentiation of Hookworms from *S. stercoralis* larvae is somewhat difficult in the parasitological methods, including the Baermann method. However, molecular diagnostics and PCR solves this problem well. In conclusion, the results showed that conventional PCR could detect all specimens that microscopically were positive.

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Authors' Contribution
AD and FJ conceived the study and designed the study protocol; AD was the supervisor of this research; FJ collected the samples, AD performed the microscopic and molecular examinations respectively, and AD was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics
Not applicable.

Conflict of Interest
The authors declare that they have no competing interests.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1. Dorris M, Viney ME, Blaxter ML. Molecular phylogenetic analysis of the genus Strongyloides and related nematodes. Inter J Parasitol. 2002.
2. Segarra-Newnham M. Manifestations, diagnosis, and treatment of Strongyloides stercoralis infection. Ann. Pharmacother. 2007.
3. Murali A, Rajendiran G, Ranganathan K, Shanthakumari S. Disseminated infection with Strongyloides stercoralis in a diabetic patient. Indian J Med Microbiol. 2010.
4. Olsen A, van Lieshout L, Marti H, Polderman T, Polman K, Steinmann P, et al. 2009. Strongyloidiasis–the most neglected of the neglected tropical diseases? Trans R Soc Trop Med Hyg. 2009.
5. Asdamongkol N, Pornsuriyasak P, Sungkanuparph S. Risk factors for strongyloidiasis hyperinfection and clinical outcomes. Southeast Asian J Trop Med Public Health. 2006.
6. Repetto SA, Durán PA, Lasala MB, González-Cappa SM. High rate of strongyloidosis infection, out of endemic area, in patients with eosinophilia and without risk of exogenous reinfections. Am J Trop Med Hyg. 2010.
7. Croker C, Reporter R, Redelings M, Mascola L. Strongyloidiasis-related deaths in the United States, 1991–2006. Am J Trop Med Hyg. 2010.
8. Lam C, Tong M, Chan K, Siu Y. Disseminated strongyloidiasis: a retrospective study of clinical course and outcome. Eur J Clin Microbiol Infect Dis. 2006.
9. Keiser PB, Nutman TB. Strongyloides stercoralis in the immunocompromised population. Clin Microbiol Rev. 2004.
10. Ericsson CD, Steffen R, Siddiqui AA, Berk SL. Diagnosis of Strongyloides stercoralis infection. Clin Infect Dis. 2001.
11. Verweij JJ, Canales M, Polman K, Ziem J, Brienen EA, Polderman AM, et al. Molecular diagnosis of Strongyloides stercoralis in faecal samples using real-time PCR. Trans R Soc Trop Med Hyg. 2009.
12. Ten Hove R, van Esbroeck M, Vervoort T, van den Ende J, Van Lieshout L, Verweij J. Molecular diagnostics of intestinal parasites in returning travellers. Eur J Clin Microbiol Infect Dis. 2009.
13. Kia E, Mahmoudi M, Zahabiun F, Meamar A. An evaluation on the efficacy of agar plate culture for detection of Strongyloides stercoralis. 2007.2(1):29-34.
14. Ashrafi K, Tahbaz A, Rahmati B. Strongyloides stercoralis: The most prevalent parasitic cause of eosinophilia in Gilan Province, northern Iran. 2010. Iran J Parasitol 5(3):40.
15. Sharifdini M, Mirhendi H, Ashrafi K, Hosseini M, Mohebali M, Khodadadi H., et al. Comparison of nested polymerase chain reaction and real-time polymerase chain reaction with parasitological methods for detection of Strongyloides stercoralis in human fecal samples. Am J Trop Med Hyg. 2015.
16. Anamnart W, Intapan PM, Maleewong W. Modified Formalin-Ether Concentration Technique for Diagnosis of Human Strongyloidiasis Korean J Parasitol. 2013;51(6):743–745.
17. Inatomi S, Kamo H, Otsuru M Suzuki T, Yoshida Y. Ova and larvae of the common helminthes of man. In: Yamaguchi T, Editor. A colour atlas of clinical parasitology. Tokyo: Wolf Medical Publication; 1981. p.

273.
18. Grove DI. Human strongyloidiasis. Advances in Parasitology 1996. 38:251-309.
19. Sharifdini M, Kia EB, Ashrafi K, Hosseini M, Mirhendi H, Mohebali M, et al. An analysis of clinical characteristics of Strongyloides stercoralis in 70 indigenous patients in Iran. Iranian J Parasitol. 2014.
20. Johnston FH, Morris PS, Speare R, McCarthy J, Currie B, Ewald D, et al. Strongyloidiasis: a review of the evidence for Australian practitioners. Aust J Rural Health. 2005.
21. Saeidinia A, Tavakoli I, Naghipour MR, Rahmati B, Lahiji HG, Salkhori O, et al. Prevalence of Strongyloides stercoralis and other intestinal parasites among institutionalized mentally disabled individuals in Rasht, northern Iran. Iran J Parasitol. 2016. 11(4):527.
22. Ghasemikhah R, Tabatabaiefar MA, Shariatzadeh SA, Shahbazi A, Hazratian T. PCR-Based Molecular Detection of Strongyloides stercoralis in Human Stool Samples from Tabriz City, Iran. Sci Pharm. 2017.
23. Sharifdini M, Keyhani A, Eshraghian MR, Kia EB. Molecular diagnosis of strongyloidiasis in a population of an endemic area through nested-PCR. Gastroenterol Hepatol Bed Bench. 2018.11(1):68-74.
24. Shokri A, Sarasiabi KS, Teshnizi SH, Mahmoodi H. Prevalence of Strongyloides stercoralis and other intestinal parasitic infections among mentally retarded residents in central institution of southern Iran. Asian Pac J Trop Biomed. 2012.
25. Ahmadi M, Beigom Kia E, Rezaeian M, Hosseini M, Kamranrashani B, Tarighi F. Prevalence of Strongyloides stercoralis and other intestinal parasites in rehabilitation centers in Mazandaran Province, northern Iran. Iran J Parasitol. 2016. 11(4): 527–533.
26. Moghaddassani H, Mirhendi H, Hosseini M, Rokni M, Mowlavi G, Kia E. Molecular diagnosis of Strongyloides stercoralis infection by PCR detection of specific DNA in human stool samples. Iran J Parasitol. 2011. 6(2):23.