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Original Article

Genetic Diversity of *Strongyloides stercoralis* with Attention to Clinical Features in Patients Originated from Three Endemic Provinces in the North and South of Iran

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Abstract

Background: *Strongyloides stercoralis* is one of the neglected tropical diseases. We aimed to verify the genetic diversity of *S. stercoralis* with attention to clinical features of the infection in patients using the *Cox*1 gene and DNA sequencing.

Methods: Using parasitological methods, *S. stercoralis* was isolated from stool samples of patients who had been referred to Tehran University of Medical Sciences, Tehran, Iran. The patients originated from three endemic provinces of Iran including Guilan and Mazandaran in the north and Khouzestan in the south of Iran. After recording the clinical symptoms of the patients, DNA extraction of the isolates, PCR, and sequencing of the *Cox*1 gene region were performed. The gene sequences were analyzed by Chromas, Bio edit, and Dna SP 6.0, and phylogenetic analysis using MEGA 7.

Results: Overall 10 isolates of *S. stercoralis* were collected from patients 55 to 73 years old. Among the patients, gastrointestinal, respiratory, and cutaneous clinical symptoms were the most common, respectively. Ten isolates were classified into 4 haplotypes, 2 of which were specific to this study. Haplotypes 2 and 3 were placed in a subclade with haplotypes including isolates from dogs in Cambodia. Haplotype 4 which is hereby introduced in the world for the first time included an isolate from a patient with hyper-infection syndrome and disseminated strongyloidiasis.

Conclusion: The *Cox*1 gene showed genetic diversity for *S. stercoralis* isolates. Accordingly, no significant genetic difference was observed between the sequences from patients with hyper-infection and non-hyper-infection. The only isolate from a patient with disseminated and hyper-infection strongyloidiasis was genetically different from all other isolates in the present study.

Introduction

Strongyloidiasis is an intestinal infection in humans caused by soil-transmitted helminth (STH) *Strongyloides stercoralis* (1). It is mainly in tropical and sub-tropical areas (2) and estimated that 613.9 million people worldwide are engaged with this infection (3). Since 2008, the WHO established a controlling program to eliminate the side effects of STH, pursuing this target, prevention and control of strongyloidiasis up to 2030, is one of the major targets for the WHO (4).

S. stercoralis has a rather unique parasitic and free-living form life cycle (5,6). In the immunocompetent person, the infection is usually asymptomatic, but an alternation in immune status will cause a rise in the burden of, if not cured well (7,8), hyper-infection syndrome, dissemination will lead to fatal patients (9). Corticosteroids possess a strong and specific relation with the development of these infections; other immunosuppressive therapies and underlying conditions can also lead to dissemination (9). This imposes a major menace to immunocompromised patients; an early laboratory diagnosis of this kind of infection has specific clinical importance.

Traditional methods for diagnosis of strongyloidiasis are based on the morphology identification of a stage larva (L3) (10). However, recently, molecular techniques have been known as a valid way to detect an organism's DNA in every parasite stage (11–13) can lead to early diagnosis and timely treatment and can be lifesaving even in cases with complex infection (14).

In the genus *Strongyloides*, *S. stercoralis*, *S. fuelleborni* and *S. fuelleborni kellyi* are mostly reported from humans (15,16). The *S. stercoralis* isolates from humans, dogs, and chimpanzees are distinct based on the *Cox*1 gene (17). Also, various investigations exist based on mitochondrial genes that demonstrate genetic differences and similarities between other geniuses properly all around the world (18–21). However, *S. stercoralis* phylogenetic studies on isolates from humans and dogs illustrated the genetic similarities between the isolates that originated from these two hosts worldwide. The zoonotic transmission theory of this infection has been supported (22).

Strongyloidiasis is endemic in the northern and southern provinces of Iran due to its appropriate climates for parasite survival (10). Increasing immune deficient diseases and corticosteroid therapy through past years created hyper-infection syndrome and death caused by *S. stercoralis* (23,24). Studies on the biology and genetics of this parasite, focusing on the patient's clinical manifestations are limited (10). Due to the genetic changes of this nematode in a host and the zoonotic importance of this parasite (22), studying the genetic variation of *S. stercoralis* based on a patient's clinical manifestations, especially the hyper-infection syndrome, and identification of its dominant genetic haplotypes is essential. Investigation of different aspects of *S. stercoralis* isolates originated from endemic regions for this parasite, can provide us with helpful information about this nematode.

Therefore, this study was conducted on the genetic diversity of *S. stercoralis* with attention to the presence or absence of hyper-infection syndrome in strongyloidiasis patients originated from three endemic provinces of Iran, including Guilan and Mazandaran Provinces in the north and Khouzestan Province in the south of the country; the analysis was based on the mitochondrial *Cox*1 gene amplification and DNA sequencing.

Methods

Sample collection

In this study in 2022 – 2023, 453 fresh stool samples were collected from 151 patients. These patients referred to the Diagnostic Laboratory of Strongyloidiasis in the School of Public Health, Tehran University of Medical

Sciences, Tehran, Iran from endemic provinces of strongyloidiasis in the north and the south provinces of Iran.

Ethics Committee of Tehran University of Medical Sciences approved the study by ethical code: IR.TUMS.REC.1402.083.

Parasitological methods

Human fresh stool samples were examined using parasitological methods including direct smear preparation, formalin-ether concentration, and nutrient agar plate culture (26). If the results of all three tests of direct smear, formalin-ether concentration and nutrient agar plate culture, were positive for *S. stercoralis*, and in direct smear and formalin-ether, and at least one larva was observed (by the microscopic magnifications 10X) and a large number of larvae of *S. stercoralis* were observed in the nutrient agar plate culture was present, the patient was considered to have a hyper-infection form of strongyloidiasis. But in other cases, such as positive only by nutrient agar plate culture or formalin-ether concentration method and nutrient agar plate culture with low parasite load, the patient was placed in the non-hyper-infection group of strongyloidiasis.

Then, after 48–72 h incubation at room temperature at about 28–30 °C, the surface of the positive nutrient agar plates culture was washed out by lukewarm phosphate-buffered saline. Then, the morphological characteristics of parasites were observed microscopically to differentiate *S. stercoralis* from other possible nematodes (27).

After confirmation, larvae of *S. stercoralis* were collected and preserved in 70% ethanol alcohol at room temperature for further use.

Molecular Methods

DNA extraction

A larva of *S. stercoralis* was isolated from each patient to extract DNA. All samples were washed with sterile distilled water to remove ethanol, followed by freezing in liquid nitrogen and thawing in boiling water for five cycles. Performed using the High Pure PCR Template Preparation Kit Sina pure (DNA KIT Cell culture, Tissues, Gram Negative Bacteria, CSF) (Lot. No: 0160102, Cat. No: Ex 6011) according to the manufacturer's instructions and stored at − 20 °C until PCR amplification was performed.

PCR amplifications and sequencing

The *Cox*1 gene of *S. stercoralis* was amplified by cox F (5′TGG TTT GGG TAC TAG TTG-3′) and cox R (5′-GAT GAG CTC AAA CTA CAC A-3′) [10].

The PCR reactions were performed in a final reaction volume of 30 µl containing 15 µL of PCR mix including 1.25 U Taq DNA polymerase, 200 µM of dNTPs and 1.5 mM MgCl2 2x red PCR Master Mix (Ampliqon, Odense, Denmark), 10 pmol of each primer and 3 µL of each sample DNA. The thermal PCR profile (Applied Biosystems 2720 Thermal Cycler, California, USA), included an initial denaturation step at 95 ˚C for 6 min followed by 35 cycles of 95 ˚C for 45 sec (denaturation), 50 ˚C for 60 sec (annealing), and 72 ˚C for 90 sec (extension), followed by a final extension at 72 ˚C for 6 min. The PCR products were run on a 1.5% agarose gel and visualized using a UV transilluminator. These PCR products were submitted to Pishgam Company and were performed using an ABI 3130xl platform (Applied Biosystems, Foster City, California, USA). The sequences were then edited, and analyzed by Chromas software version 2.6.1 (South Brisbane, Australia) and Clustal W method using Bio edit software version 7.1 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and then they were compared with the sequences present in GenBank database.

Phylogenetic analysis

A phylogenetic tree was constructed using the Maximum Likelihood algorithm and Tamura-3-Parameter option by MEGA7

software (28). Bootstrap analysis was done based on 1,000 replications.

Neutrality and diversity indices

To calculate the number of Polymorphism sites, Nucleotide diversity (π), Haplotypes diversity (Hd± SD) and the number of Haplotypes for *S. stercoralis* isolates, Dna SP 6.0 software was employed (29).

Results

Ten isolates of *S. stercoralis* were obtained from patients originated three provinces (Guilan, Mazandaran and Khuzestan) from endemic areas. However, during this study no patient was referred from the Hormozgan Province.

Based on parasitological methods the load of six chronic strongyloidiasis, three hyper-infection patients and a hyper-infection and disseminate were diagnosed. The isolates and the province of origin of each patient and the corresponding accession number which was submitted in GenBank are shown in Table 1.

Table 1: List of *S. stercoralis* (S1 to S10) isolates with clinical symptoms and demographic characteristics of patients and the related accession numbers in GenBank based on amplification of *Cox*1 gene and haplotyping by Dna SP6.0

Isolate	*Accession number in GenBank*	*Age*	*Male/female*	*Patient original province*	*Haplotyping by Dna SP6.0*	*chronic strongyloidiasis / hyper- infection /disseminated*	*clinical symptoms*	*Eosinophilia %*
S1	OR552505	71	Male	Guilan	1	hyper- infection	gastrointestinal	-
S2	OR552506	60	Male	Guilan	2	hyper- infection	gastrointestinal	-
S3	OR552507	73	Male	Guilan	3	hyper- infection	gastrointestinal , pulmonary	6
S4	OR552508	55	Male	Mazandaran	3	chronic strongyloidiasis	gastrointestinal and pulmonary	-
S5	OR552509	73	Male	Guilan	1	chronic strongyloidiasis	gastrointestinal and pulmonary	-
S6	OR552510	69	Male	Khuzestan	2	chronic strongyloidiasis	gastrointestinal	15
S7	OR552511	63	Male	Guilan	1	chronic strongyloidiasis	gastrointestinal , pulmonary and cutaneous	26
S8	OR552512	70	Female	Mazandaran	1	chronic strongyloidiasis	gastrointestinal , pulmonary	34
S9	OR552513	71	Male	Khuzestan	4	disseminated and hyper- infection	gastrointestinal	-
S10	OR552514	56	Female	Guilan	2	chronic strongyloidiasis	-	32

After optimizing PCR conditions in terms of materials and thermal PCR protocol and the number of cycles, all *S. stercoralis* isolates successfully demonstrated the amplification of about 509-bp target band for the *Cox*1(Fig. 1).

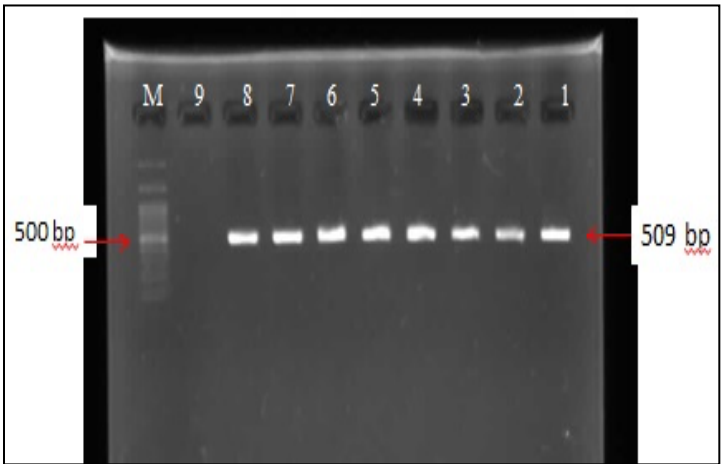

Fig. 1: Agarose-gel electrophoresis of polymerase chain reaction (PCR) products amplified for *Cox* 1 gene of *Strongyloides stercoralis* samples. Lanes 1 to 7: S. *stercoralis* samples; Lane 8: Positive control (*S. stercoralis* larva); Lane 9: Negative control; and Lane M: 100-bp DNA ladder

BLAST analysis indicated that, among these isolates, there was 100% homology between OR5525014 and OR552506 isolates from Guilan Province and OR5525010 from Khuzestan; and also 100% homology among OR552507 from Guilan Province and OR552508 sequences from Mazandaran Province. Also, OR5525012 from Mazandaran Province, OR5525011, OR552505 and OR5525009 from Guilan Province had 100% homology, but the isolate from Khuzestan Province OR552513 had no homology with other isolates.

Inter-species and intra-species genetic variations between present isolates of *S. stercoralis* in this study were calculated. The inter-species genetic variation between *S. stercoralis* ranged from 0 to 0.023%. Furthermore, the pairwise distance indicated the presence of intra-species genetic variation of the current isolates of *S. stercoralis*, and 0 to 0.03% of those with the sequences previously available in GenBank.

The genetic variation between *S. stercoralis* isolates ranged from 0 to 0.023%. The pairwise distance analysis also revealed the existence of intra-species genetic variation in the current isolates, which was comparable to that of previously available sequences in GenBank (0 to 0.03%). Phylogenetic trees of these 10 isolates along with some other isolates from GenBank were constructed by applying MEGA7 software (Fig. 2). Based on this tree, all isolates of this study were put in one major clade. The tree revealed that the ten sequences of *S. stercoralis* isolates in the study consisted of four distinct haplotypes, which were placed into one clade and three subclades.

The tree indicated that the ten sequences of *S. stercoralis* isolates in the present study consisted of four haplotypes, placed into one clade and three subclades.

Genetic diversity and neutrality

With regards to the results of Dna SP6.0 analysis, the number of Haplotype (Hn) was 4 and Haplotype diversity (Hd± SD) was 0.0778±0.091. Also, Nucleotide diversity Nd (π) was 0.00936.

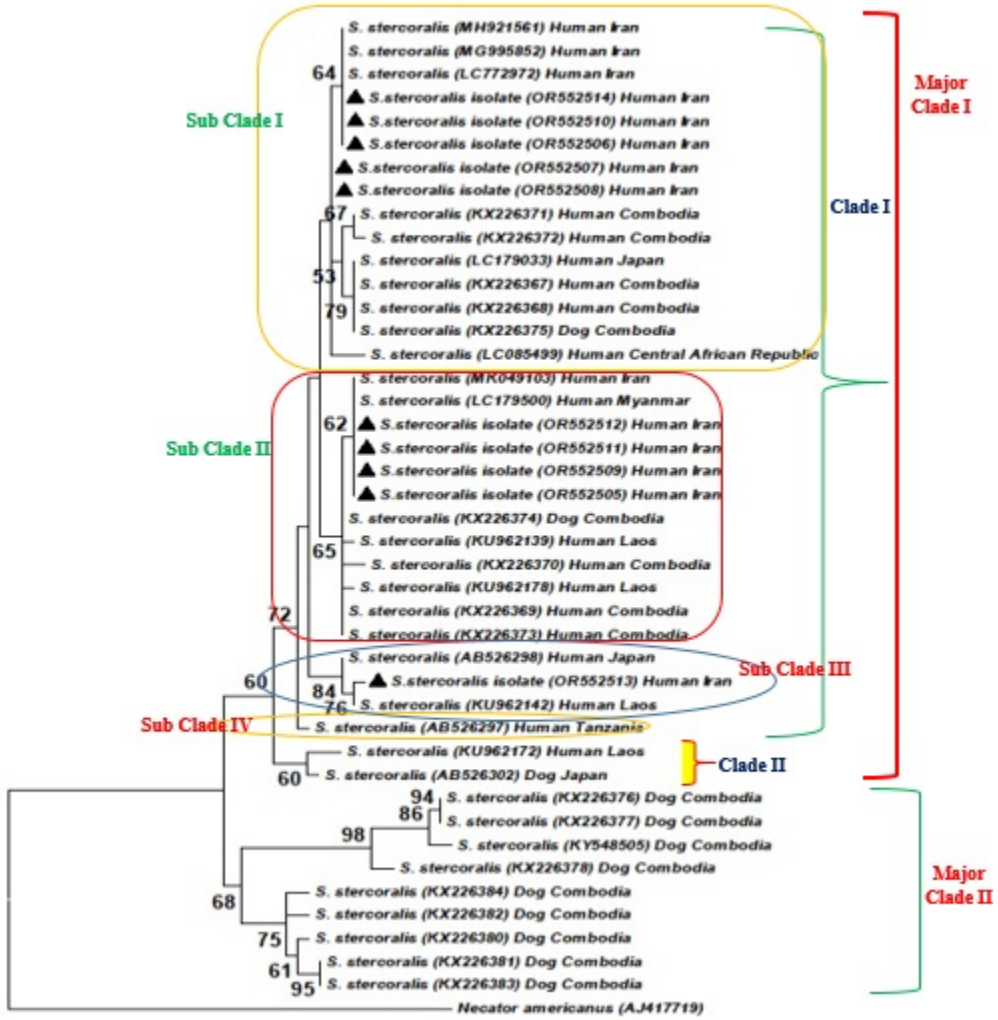

Fig. 2: Phylogenetic tree of *Strongyloides stercoralis* (▲) isolates obtained in this study and reference sequences retrieved from GenBank based on *Cox*1 gene sequences and constructed tree using Tamura 3-parameter model by MEGA software version 7.0. *Necator americanus* (AJ417719) was selected as an out-group

Discussion

Since 2008, the WHO has started a program to control and eliminate complications caused by strongyloidiasis (4). The symptoms of strongyloidiasis, depending on the immune system, vary from no symptoms, hyper-infection syndrome to disseminated infection, which in most cases are fatal in case of delay in treatment (7,8). DNA-based techniques have created great tools to identify *Strongyloides* spp. accurately without considering the developmental stage. In endemic regions of the world, studies have been conducted on the genetic diversity of *S. stercoralis* from humans (18,30–32). The latest studies show that there can be different haplotypes from isolates of *S. stercoralis* species in a host (22,33).

In the past years, with the increase in immune system deficiency diseases and corticosteroid treatments, we have seen cases of hyper-infection syndrome caused by *S. stercoralis* and deaths caused by this parasite (23,24). Studies on the biology and genetics

of this parasite focusing on clinical symptoms of the patients in the world are very limited. Due to genetic changes of this nematode in a host and the zoonotic importance of this parasite (22), in this study, for the first time in Iran, the genetic diversity of *S. stercoralis* based on the clinical symptoms of the patient, especially the hyper-infection syndrome, and the identification of the dominant genetic haplotypes based on the *Cox*1 gene were examined.

In the present study, 10 patients from endemic areas in the north (Guilan and Mazandaran Provinces) and south (Khuzestan Province) of Iran were examined. 6 patients (60%) had strongyloidiasis without hyper-infection, 3 out of 4 patients (40%) had hyper-infection and one had hyper-infection and disseminated strongyloidiasis. The frequency of clinical symptoms in these patients included gastrointestinal, pulmonary, and cutaneous, respectively, similar to the other study in Iran (34). In this regard, Nilforoushan et al had only one difference with our study in case of the order of importance: gastrointestinal, cutaneous and respiratory symptoms (27). Of course, only one patient showed cutaneous symptoms in the form of itching, but Larva Currens symptoms were not reported in any cases, similar to other studies in Iran (25,27,34).

In the present study, the range of eosinophilia was recorded between 6 to 34%; but in another study in Iran, there was found difference significant between age and range of eosinophilia, so the rate of eosinophilia reduced with the increase in age (25).

This study found that 6 out of the 10 patients had chronic strongyloidiasis, 3 had hyper-infection, and one had both hyper-infection and disseminated strongyloidiasis infection. Additionally, 7 patients had underlying medical conditions such as diabetes, kidney disease, multiple myeloma, heart failure, hypothyroidism, lung disease, and CLL. All patients with hyper-infection or disseminated infection had an underlying condition. The significance of the presence of underlying disease has been reported in prior literature as well (25,34).

Pairwise distance of the current study isolates showed that intra-species genetic variation within *S. stercoralis* nucleotide sequences was 0 to 0.023 %. This variation was lower than variations reported in similar studies conducted in Iran (31). According to other studies, the Pairwise difference in the *Cox*1 gene among interbreeding strains of a nematode species was reported to be usually less than 6%. This difference between distinct species in a genus was more than 10% (35). Regardless of the host and locality of the isolates, the pairwise difference in nucleotide sequence of the *Cox*1 gene among isolates of *S. stercoralis* from humans, apes and dogs was less than 4% (36).

The nucleotide variation of the genome is characterized by changes in protein expression and phenotypes of organisms, which may result in expected changes in the protein structure and biological effects (35). By DNA SP6. analysis, 10 *S. stercoralis* isolates of the current study included 4 haplotypes, 2 of which were specific to this study. Also, in another study in Iran, the phylogenetic tree indicated the presence of 5 haplotypes among 10 sequences of *S. stercoralis* available (31). In the present study, all of the investigated isolates were placed in the same major clade as human or dog isolates from different parts of the world.

But, in the subclade II, the sequences of the current study including OR552512, OR552511 and OR552509, infected with chronic disease, and OR552505, infected with hyper-infection, with human isolates (MK049103) from north of Iran and Myanmar (LC179500) were grouped with human isolates from Laos (KU96139 and KU962178), Cambodia (KX226369, KX226370, and KX226373) and with dog isolate from Cambodia (KX226374). Also, Human isolates of *S. stercoralis* based on the *Cox*1 gene have been reported in different

studies homology with isolates of dogs from different parts of the world (31,34). All studies mentioned above and other studies from different parts of the world focused on the similarity of this *Cox*1 gene of human *S. stercoralis* isolates and isolates from dogs, showing the importance of the zoonosis of this parasite.

In the present study, OR552505, OR552506 and OR552507 isolates from Guilan Province were related to patients with hyper-infection. All patients were male and had an underlying disease that was genetically placed in three separate haplotypes in the phylogenetic tree along with other isolates collected from non-hyper-infection patients, but the OR552513 isolate was collected from a male patient from Khuzestan, suffering from disseminated and hyper-infection form of strongyloidiasis with underlying CLL disease. This isolate was included in a specific haplotype in this study and genetically did not have 100% homology with any isolates in the present study and human and dog isolates from other parts of the world; similar studies, conducted in Iran, based on this gene reported that haplotype as a haplotype with high pathogenicity in male patients of Mazandaran Province (34). Although the present study was the first study in the field of genetic diversity of human isolates of *S. stercoralis,* based on the *Cox*1 gene sequence analysis in Iran, focusing on the clinical symptoms of patients, future studies with a larger sample size from all the endemic provinces of this disease and the use of other genes to detect new haplotypes of this parasite is required and finding the relationship between the existence of a specific haplotype and the presence and absence of disseminated and hyper-infection syndrome of strongyloidiasis is needed.

Conclusion

The need for speculation on the host factors is raised. Therefore, for future studies, investigation into the role of different predisposing conditions in the onset of hyper-infection syndrome and disseminated strongyloidiasis in the patients is recommended.

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Conflict of interests

The authors declare that they have no competing interests.

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