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Short Communication

Trichostrongylus colubriformis: Possible Most Common Cause of Human Infection in Mazandaran Province, North of Iran

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Abstract

Background: Infection with *Trichostrongylus* spp. is common among human and herbivorous in most parts of Iran, especially in southern and northern areas. The aim of present study was to identify *Trichostrongylus* spp. among human population using excreted egg specimens, by the molecular method, in Mazandaran Province, northern Iran.

Methods: Overall, 33 positive fecal specimens were randomly sampled and examined. PCR amplification of ITS2-rDNA region was performed on the isolated egg and then a restriction fragment length polymorphism (RFLP) profile was considered to discriminate of *Trichostrongylus* spp.

Results: A total of 33 positive fecal specimens, 29(78.9%), 4(12.1%) were found *T. colubriformis* and *T. axei* respectively. Our data appear the molecular evidence of both human *T. colubriformis* and *T. axei* infections in North of Iran.

Conclusion: *T. colubriformis* was the probable most common zoonotic species causing human trichostrongylosis infection in the area

Introduction

The genus *Trichostrongylus* is mostly known as animal parasites and occurs throughout the world. Human and herbivores infections with *Trichostrongylus* spp. have been reported from different parts of the world (1, 2). Trichostrongylosis is caused by more than 30 species. Most of them are parasitic in herbivores, although 10 species have

also been reported from humans. Humans become infected with *Trichostrongylus* spp. mainly by ingesting infective-stage larvae or infrequently penetrating to the skin. Human cases have been reported from many countries, including Iran, Japan, Thailand, South Korea, China, United States, and Australia(2– 4). In the laboratory, *Trichostrongylus* infection is frequently diagnosed by detection of eggs in fecal samples (5). Human and herbivores animals can be infected by several species of *Trichostrongylus* parasites. Thus, detailed discrimination of *Trichostrongylus* spp. is important for efficient prevention and control programs of the parasite (2, 4, 6, 7).

However, conventional morphological methods for classification of *Trichostrongylus* spp., are relatively reliable for *Trichostrongylus* males, they are laborious and cannot be relevant for identification of females and eggs (3, 4). Diagnosis of trichostrongylosis is performed mostly and routinely by finding the eggs in fecal samples or producing third-stage larvae by culturing of fecal samples. However, the eggs are not mainly differentiable morphologically at the level of species and culturing process is very time consuming. Also, the egg morphology of *Trichostrongylus* and hookworm species is relatively similar, and it is difficult to differentiate them (5,8-10). Most investigations have showed molecular methods (particularly ITS2 region in rDNA gene) as valuable tools to differentiate *Trichostrongylus* species (9-13). In addition, the progress of highly sensitive diagnostic methods for the accurate typing of individual eggs of helminth species is extremely important to the control of helminthic infections among human and animals. In Iran, nine species of *Trichostrongylus* have been reported from different areas. Among them, *T. orientalis* and *T. colubriformis* were detected more frequently in humans just by the morphological methods (3,6-8,14). However, eight species (except *T. orientalis)* in ruminants have been recognized in Iran by the molecular methods (7, 8). Considering to the climate and socio-economic conditions, environmental situation and human lifestyle in rural areas, north of Iran is considered as an endemic area of trichostrongyliasis. Mazandaran Province is located in the north of Iran (53°6' E, 36°23'N). Most of the rural residents in this area are paddy field workers and livestock husbandry that can expose to the different zoonotic parasitic infections including *Trichostrongylus* spp (3, 6, 15).

Due to the little information is available about the molecular typing of *Trichostrongylus* spp among infected human in Mazandaran Province since 1975 (3), this study was carried out to typing *Trichostrongylus* spp. infections among infected human individuals using egg sample of the parasite, in the area.

Materials and Methods

Sample collection

The egg specimens of *Trichostrongylus* were randomly collected, by direct, *formalin-ether and* floatation methods, from 33 fecal specimens of infected subjects who referred to Medical diagnostic laboratories from different parts of the Mazandarn Province, northern Iran. Fecal samples were transferred to the Research Laboratory of parasitology in Sari, department of parasitology. The isolated egg samples were preserved in absolute ethanol and stored at -20 °C. For the genomic DNA extraction, a number of eggs of *Trichostrongylus* were rinsed several times with distilled water to remove the ethanol prior to DNA extraction.

DNA extraction

A total of 33 fecal positive samples were chosen for DNA extraction and amplification of ITS2-rDNA. *Trichostrongylus* genomic DNA was extracted from each fecal samples contains a number of eggs, using DNA mimi Kit (Bioneer, Korea) according to manufacturer's instruction. Approximately 200 µl packed volume of eggs was mechanically grinded in 180 µl lysis buffer and then 20 µl proteinase K was used, incubated at 55 °C for 2 hours and terminated with 10 min incubation at 95 °C to

inactivate the proteinase K. The pure DNA was eluted in Tris-HCl buffer by effective washing and stored at -20 °C. The concentration of DNA was determined using Nanodrop machine. Totally, 33 egg isolates were used for DNA amplification and polymerase chain reaction (PCR)-RFLP analysis.

PCR assay

Trichostrongylus genomic DNA were analyzed by PCR of rDNA internal transcribed spacer 2 (ITS2-rDNA) and PCR-restriction fragment length polymorphism (PCR-RFLP) as described previously for *Trichostrongylus* spp (11, 16 -18). To amplify ITS2 region of rDNA and sequences, the PCR were performed by oligonucleotide primers NC1: 5'-ACGTCTGGTTCAGGGTTGTT-3' (forward) and NC2: 5'-TTAG TTTCTTTTCCTCCGCT-3' (reverse). DNA amplification was performed in a final volume of 20 µl containing 5µl DNA template (200 ng/µl), 10 mM Tris- HCl buffer (pH, 9.0), 500 mM KCl, 2.5 mM Mg Cl_2, 2.5 mM of each dNTP, 25 25pmol/ul of each primer (NC1 and NC2), 1.5 unit Taq polymerase in reaction buffer. The PCR conditions for each isolates were as follows: an initial denaturing (1 cycle 94 °C for 10 min), followed by 35 cycles denaturation (94 °C for 30s), annealing (55 °C for for 30s), extension (72 °C for for 30s) and final extension (72 °C for 5 min). After amplification the PCR products were electrophoresed through 1.5% (w/v) Tris-Borate - EDTA (TBE) agarose gel and stained with ethidum bromide to visualize the separated DNA bands. Positive and negative controls were included in each PCR reaction.

ITS2-RFLP and sequencing

The PCR products of each isolates were digested separately for 24 hours (overnight) with two base cutting restriction enzymes of *DraI* and *Hinf1* using 10x assay buffer as recommended by the manufacturer (Fermentase, Lithuania). The digestion by all restriction enzymes were performed by incubating 5µl PCR product with 1.5 µl assay buffer, 6 µl sterile distilled water and 0.5 µl restriction enzymes (8-10 U/ µl) at 37 °C. The DNA fragments were separated by electrophoresis through 2% (w/v) TBE agarose gel (50-100 mV constant voltage). The ethidium bromide stained bands were detected on Gel Doc (Mini-SUB with power Pac Basic, BioRad), and the sizes of PCR products and restriction fragments were analyzed using the UV doc images software package. Moreover, the five PCR products were sequenced. The sequences were aligned and compared with the sequence data for *Trichostrongylus* spp. in GenBank.

Results

The region ITS2-PCR and linked ITS2-PCR-RFLP were used to characterize genotypes of *Trichostrongylus*. DNA isolated from eggs recovered from all fecal samples. The ITS2-PCR amplified with NC1 and NC2 primers yielded unique bands the size about 330bp, a similar size to those obtained with the universal *Trichostrongylus* spp. (Fig.1). The PCR product after digestion of the ITS2 fragments with *DraI* and *Hinf1* enzymes showed two clearly distinguishable patterns of *Trichostrongylus* spp.

Overall, 33 egg isolates based on the PCR-RFLP, two species including *T. colubriformis* 29(78.9%) and *T.axei*4 (12.1%) were identified. Two different fragments were produced with *Hinfl* enzyme in *T. colubriformis* (about 90bp and 238bp, respectively). Similarly restriction with *DraI* enzyme produced two different fragment size in the PCR product of *T.colubriformis* was 238, 90 bp and *T.axei* 218,110 bp respectively. However, there were found differences between species in their RFLP-ITS2 patterns (Fig. 3). The unrestricted PCR products were of a similar size (about 330 bp) for all 2 species (Fig. 1). However, their restriction patterns were different in these species (Fig 2. and Fig 3.). Conversely, the difference between the two species in their restriction patterns is observed.

Trichostrongylus ITS2- rDNA products of five *T. colubriformis* were also sequenced and were found 100% homology with reference sequence (X78063) and one Iranian animal isolate (JF276020) and human isolates from Sari in Mazandaran Province (KF989494-7) for *T. colubriformis*. The sequences were identical to those of the *T. colubriformis* sequence in GenBank.

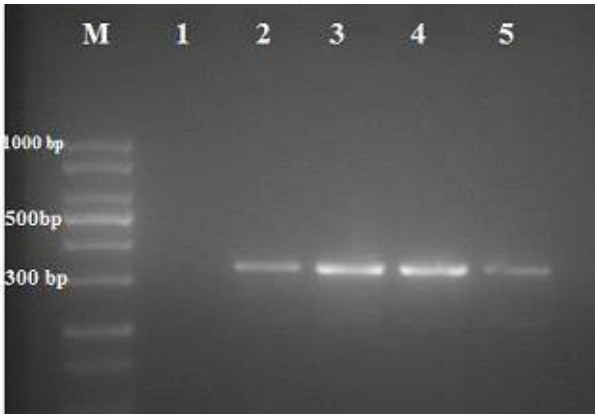

Fig. 1: Gel electrophoresis of primary PCR products of *Trichostrongylus* isolates. Lane 2-5 *Trichostrongylus* isolates, Lane 1: negative control, M: DNA marker.

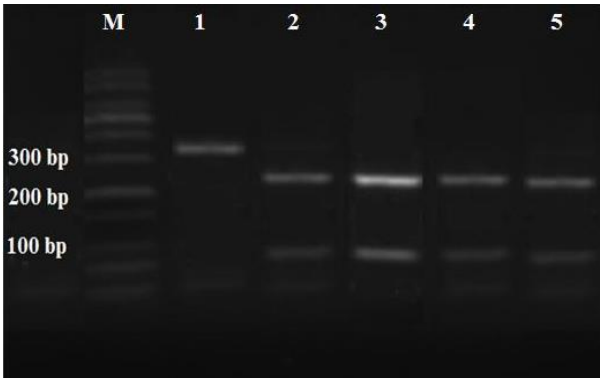

Fig.2: *Trichostrongylus* species identified by PCR-RFLP of ITS2 Region of rDNA gene using *Hinf1*enzyme. Lane 2-5 *T. colubriformis* (238bp, 90bp), M: 100bp DNA marker).

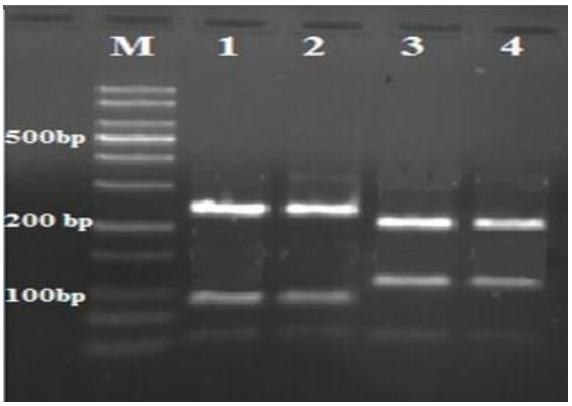

Fig.3: *Trichostrongylus* species identified by PCR-RFLP of ITS2 Region of rDNA gene using *Hinf1*enzyme. Lane 1-2 *T. colubriformis* (238bp, 90bp), Lane 3-4 using *Dral* enzyme *T. axei* (218bp, 110bp), M: 100bp DNA marker).

Discussion

The results of our preliminary study provide new information about the status of the *Trichostrongylus* species in human from Mazandaran Province, north of Iran. Our study showed the most 29(78.9%) of positive fecal specimens, were found *T. colubriformis* and the remaining 4(12.1%) *T. axei*.

Based on the pervious morphology and molecular studies upon sequence variation within the ITS2 region in the nuclear ribosomal gene cluster, the occurrence of *T. orientalis*, *T. colubriformis*, *T. vitrinus*, *T. axei*, *T. capricola*, *T. probolurus*, and *T. skrjabini* with more frequency of *T. orientalis* and *T. colubriformis* have been previously reported in different animals (sheep, goat, cattle, camel and buffalo) in Iran (7, 8- 10). In our study, parasite sequences also showed more than 95% homology with the main haplotype observed in humans in rural areas of Laos (16). In human, among seven species of *Trichostrongylus*, six species including *T. orintalis, T. capricola, T. lerouxi, T. vitrinus, T. axei* and *T. colubriformis* have previously been reported more frequently from different parts of Iran by morphological and molecular methods (3, 6, 19-21).

In countries, such as Iran, where humans were infected with *T. colubriformis,* this parasite was also common in domestic animals such as ruminants (3,7-10). Thus, *T. colubriformis* was thought to be the main zoonotic species. Consequently, at least in Mazandaran Province, molecular evidence confirms *T. colubriformis* as most possible common cause of human infection. Moreover, sequencing data from human fecal isolates, showed 100% homology with known sequences for *T. colubriformis* from sheep and human isolates from Mazandaran Province. Therefore, it appears that the sheep could be the main source for contaminating the vegetables and water in this region. Moreover, due to lack of intraspecific variation in the ITS-2 region of *T. colubriformis* ribosomal DNA, it could be useful for distinguishing between species of Trichostrongylus (18).

On the other hand, *T. axei* were thought to be less common species in human. Human infection of *T. axei* has been reported in Siberia, Armania and Pakistan in the past literatures and Iran. In addition, *T. orientalis* was considered as a mainly specific human parasite. In the past (about 30 years ago), *T. orientalis* was documented, only using coprological examination, as the common human species in north of Iran (3), because human faeces utilized as a fertilizer by farmers. Over the last few decades, the species have not been reported from human in the area.

We explain the direct PCR on egg specimens with high sensitivity. Direct PCR on the individual eggs isolated from fecal infected person is a suitable approach, because it is rapid, labor effective, and can be used to generate template from minute quantities of materials. This is especially important, because in infected human individuals often only egg specimens are more convenient and more available. Furthermore, unfortunately, it is often difficult to isolate adequate, pure DNA from some worms for example nematode species, because of their rough cuticle, and a 'white flocculate' substance found to co-precipitate with DNA during isolation (22), which inhibits PCR reactions or causes amplification of non-specific products. In this paper, we describe a DNA isolation method, which prevents of these problems and a PCR technique, which is sufficiently sensitive to sequence rDNA from *Trichostrongylus* eggs.

Conclusion

T. colubriformis was the probable most important and common zoonotic species causing human *Trichostrongylosis* in the area. As a whole, hygienic advices for use of organic fertilizer must be distributed on a large level. It is also mandatory that fresh vegetables be washed carefully and thoroughly before ingestion, and only dried manure should be used as fertilizer.

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