

Molecular characterization of *Hymenolepis nana* based on nuclear rDNA ITS2 gene marker

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Abstract

Introduction: *Hymenolepis nana* is a zoonotic tapeworm with widespread distribution. The goal of the present study was to identify the parasite in the specimens collected from NorthWestern regions of Iran using PCR-sequencing method.

Methods: A total of 1521 stool samples were collected from the study individuals. Initially, the identification of *Hymenolepis nana* was confirmed by parasitological method including direct wet-mount and formalin-ethyl acetate concentration methods. Afterward, PCR-sequencing analysis of ribosomal ITS2 fragment was targeted to investigate the molecular identification of the parasite.

Results: Overall, 0.65% (10/1521) of the isolates were contaminated with *H. nana* in formalin-ethyl acetate concentration. All ten isolates were successfully amplified by PCR and further sequenced. The determined sequences were deposited in GenBank under the accession numbers MH337810 -MH337819.

Conclusion: Our results clarified the presence of *H. nana* among the patients in the study areas. In addition, the molecular technique could be accessible when the human eggs are the only sources available to identify and diagnose the parasite.

Keywords: *Hymenolepis nana*, rDNAITS2, PCR, Iran.

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Introduction

Hymenolepis nana, generally known as the dwarf tapeworm, is one of the most common tapeworms of humans and rodents, in which the parasite can cause hymenolepiasis. This zoonotic tapeworm has a cosmopolitan distribution with socio-economic and medical significance which may occur in many countries, worldwide^{1,2}. The parasite is among the neglected tropical diseases (NTD). Human

hymenolepiasis caused by one of two adult tapeworms *Hymenolepis diminuta* or *Hymenolepis nana*, is a globally widespread zoonotic infection known to be endemic in Asia, Southern and Eastern Europe, Central and South America, and Africa^{3,4,5}. *H. nana*, however, accounts for the most common cause of all cestode infections in humans and in temperate zones with high incidence in children and institutionalized groups^{6,7}. Although the extent of clinical manifestations depends on the worm burden yet the infection with *H. nana* usually causes many clinical symptoms such as headache, weakness, anorexia, abdominal pain, and diarrhea⁸. Infection is usually self-cleared by adolescence and is infrequent in healthy adults⁷. The drug of choice to treat hymenolepiasis is praziquantel which is more effective than other drugs including mebendazole or niclosamide⁹.

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H. nana is the only cestode capable of completing its life cycle in the final host without involving an intermediate host. It is also able to complete its entire cycle in a single host (auto-infection)¹⁰. The worm could be easily renewed by new generations and complete their life cycle only in human intestine. The issue may cause epidemics in close proximity and compact environments such as childcare institutions, dorms, and barracks¹¹. Contamination is most commonly acquired from eggs in the faeces of another infected individual, transferred by contaminated food¹², although an indirect life cycle utilizing insects as intermediate hosts may occur¹⁰. Expulsed eggs in human faeces have an important role in diagnosis as they are used to identify morphological features¹³. However, with advancement in the field of molecular biology, techniques such as polymerase chain reaction (PCR) has provided simple and rapid procedure in identification of parasites. This approach is a widely used method for the accurate differentiation and characterization of helminthic parasites including the cestodes^{14,17}.

Up to now several studies have reported the presence of *H.nana* infection in Iran with prevalence rates ranging from 0.01% to 10% in different part of the country¹⁸⁻²², although these researches mainly focused on morphological method. Therefore, there was a need for a precise and sensitive method that could more accurately detect the parasite. So the main objective of the present study was to molecular characterization of *H. nana* in Qazvin province, North West of Iran.

Materials and method

Sample collection and processing

A total of 1521 stool samples were collected from the individuals, referred to Shahid Bolandian health care center in Qazvin province, situated in Northern border of central Iran, to be routinely examined for the presence of ova and parasites in 2016. Ethical approval of the study was obtained from the Medical Ethics Committee of Qazvin University of Medical Sciences (IR. QUMS. REG. 1396. 293). All specimens were surveyed in the parasitology laboratory at Qazvin Medical School. Diagnosis of *H. nana* was confirmed by direct wet-mount and formalin-ethyl acetate concentration methods.

DNA extraction and PCR amplification

Genomic DNA was extracted by QIAamp DNA Stool Mini Kit. (QIAGEN, Germany). The internal transcribed

spacer 2 (ITS2) region of ribosomal DNA (rDNA), was amplified using specific primers of *H. nana* as follows: Forward primer (ITS2F): 5' GTGAATCGCAGACT-GCTTTG 3'

Reverse Primer (ITS2R): 5' CTGAGGTCAG-GTCTTCCATAC 3'

Twenty microliter reaction volume containing a ready-made mixture of Amplicon (Taq DNA Polymerase Master Mix RED, Denmark) with template DNA, 0.1 μM of each primer and distilled water were used for PCR under following temperature conditions: 95 °C for 5 min; 35 cycles of 95 °C for 20 s, annealing step at 59 °C for 20 s and 72 °C for 30s; final extension cycle of 72 °C for 5 min. Subsequently, the PCR product was stained and electrophoresed on a 2% agarose gel in TBE buffer (Tris, boric acid and EDTA). The specific band appeared under UV light.

Sequencing analysis

All PCR products were purified and sequenced with ABI 3130X sequencer. The resulting sequences were adjusted manually by chromas (version 1.0.0.1), and then compared with available reference sequences in BLAST GenBank database by BLAST software available at (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All *H. nana* sequences were submitted to the NCBI database under the Accession Nos. MH337810- MH337819.

Phylogenetic analysis

ClustalW incorporated in the BioEdit software was used to sequence alignment (Hall1999). Phylogenetic tree was constructed by the MEGA7 software (Molecular and Evolution Genetic Analysis v6), Maximum-Likelihood algorithm with Tamura-3 parameter substitution model was applied. Finally, a bootstrap number of 1000 replicates was considered.

Statistical method

Chi-square test using SPSS software version 17.00 (SPSS Inc., Chicago, IL, USA) was used to determine signification of variables. A P value of <0.05 was considered significant for differences, statistically.

Results

Out of 1521 stool samples, 10 (0.65%) isolates were contaminated with *H. nana* in formalin-ethyl acetate concentration (Fig. 1).



Figure1: The egg of *H.nana* in stool examination with ×400 magnifications (These pictures were originally captured from the current study specimens.)

PCR-amplification of the rDNA ITS2 region yielded a single band of approximately 530 bp for *H. nana* (GenBank accession number MH337810 -MH337819) (Fig. 2).

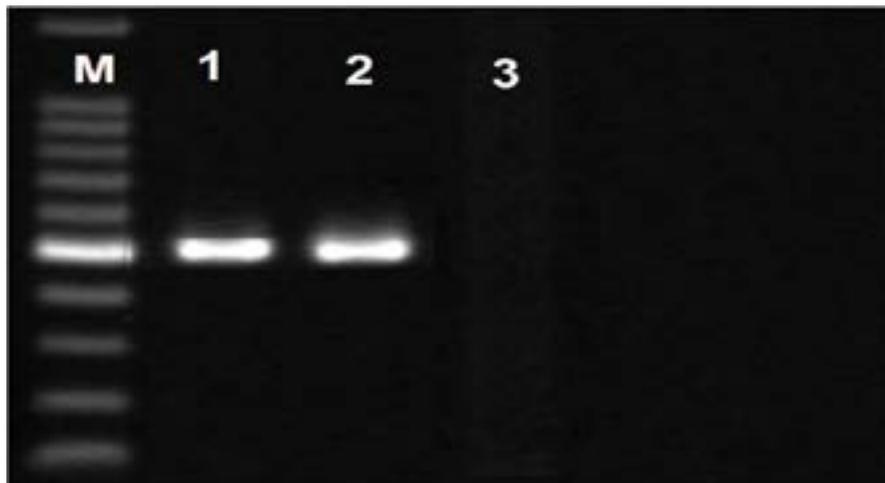


Figure2: PCR patterns of *H.nana* obtained from patient's samples. Lane M 100-bp size marker, Lane 1 and 2 *H.nana* (530 bp), Lane 3 Negative control

To determine the inter-relationship between *H. nana* and their taxonomic correlation and other members of the family *Hymenolepididae*, phylogenetic trees were constructed taking *Taenia hydatigena* as the outgroup (Fig. 3).

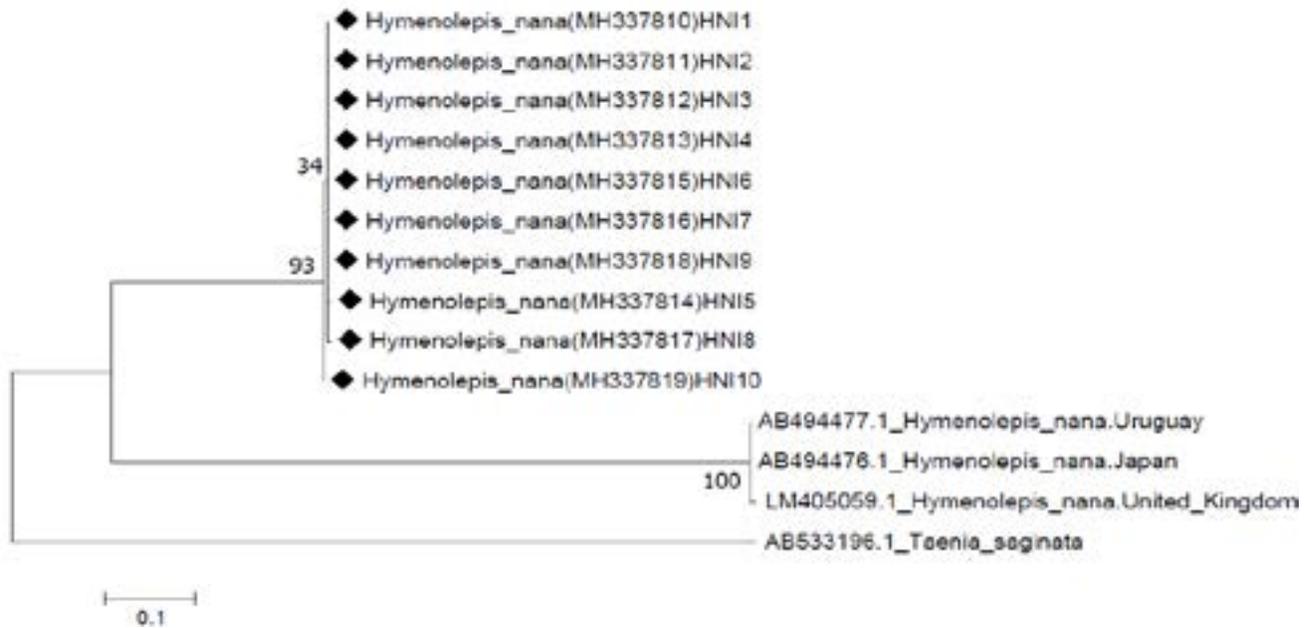


Figure 3: Phylogenetic relationships among *H. nana* taxa inferred by ML methods. The phylogeny inferred from the ML analyses showed identical tree topologies with strong bootstrap values. The overall tree structure included two clusters. *H. nana* was clarified in present study (◆), a close intra-specific proximity was seen among the isolates of *H. nana* obtained from different isolates including the isolates of our study and also the isolates of other countries.

The highest infection rate was observed in the age group under 30 years old. The results of clinical signs showed abdominal pain was the most frequent gastrointestinal symptom in the people infected with *H. nana* who had digestive tract symptoms among those the most common symptom was diarrhea.

The phylogeny inferred from the ML analyses showed identical tree topologies with an informative clading pattern with strong bootstrap (1000 replication) values. The overall tree structure included two clusters, labeled as *H. nana* clades. In these clades, a close intra-specific proximity was seen among the isolates of *H. nana* obtained from different isolates including the isolates of our study and also the isolates of other countries.

Discussion

Human hymenolepiasis, a disease that occurs throughout the world, is usually produced by two species of *Hymenolepis*—*H. nana* and *H. diminuta*³. Diagnosis and identification of these tapeworms in human patients is usually based on egg morphology in fecal examination¹³. However, the identification based on morphology has not been a suitable method in determining the species of tapeworm due to the similarities present in the morphological and

phenotypic profile of different stages of the parasite eggs²³. In the current study, we used PCR-sequencing technique for characterization of nuclear rDNA ITS2 gene sequences of *H. nana* to precisely identify the parasite. So far, different studies have been carried out in Iran where *H. nana* infections have been reported. Kheirandish^{18,19} and Badparva et al²⁰ in three different studies conducted to determine the prevalence of intestinal parasites in Western Iran, reported *H. nana* infection rates of 0.1%, 0.5%, and 0.3%, respectively¹⁸⁻²⁰. Another study in Northern Iran found an infection rate of 1.9% caused by *H. nana* among the food handlers of Sari, the capital of Mazandaran province²⁴. In a study on 800 school children in Golestan province, northern Iran, *H. nana* infection rate was 1.5 %²⁵. Similarly, in a study performed in rural region of Orumiyeh, northwest of Iran, the infection rate of the parasite in primary school children was 0.2%²². Likewise, in two different studies carried out in southwestern Iran the infection rates by *H. nana* were estimated at 1.29% (2012) and 0.04% (2017)^{26,27}. Lastly, in a study from Mashhad, northeast Iran, the infection rate caused by *H. nana* among HIV positive individuals (2010) was reported around 10%. In the current study, the infection rate of *H. nana* parasite was 0.6% while in a

previous study conducted in Qazvin province in 2015 it was 0.01%²⁸. This difference in infection rate may be due to reasons such as the difference in sample size, sampling sites, low sensitivity of the diagnostic method, and finally the use of single stool examination method. Infections with *H. nana* in humans are ubiquitous, particularly in children of developing countries²⁹⁻³¹. In a study reported from Thailand, stool examination was performed on 2083 Thai children from orphanages and primary schools. The worm infection was only found in children from orphanages with a prevalence rate of 13.12 %⁸. Also, the infection rate in children of Ethiopia, Mexico, and Pakistan was detected at 28.3%, 1.5%, and 1.8%, respectively³²⁻³⁴. It is worth mentioning that *H. nana* is the most common cestode of humans, particularly in young individuals and mainly in children^{35,36}. In the present study, the highest infection rate was observed in the age group under 30 years old, a finding consistent with that of a previous study on this parasite^{8,32,34,37,38}.

H. nana usually causes many clinical symptoms such as headache, weakness, loss of appetite (anorexia), meteorism (bloating, nausea, vomiting, itching, irritability, sleeplessness, enuresis, abdominal pain, and diarrhea)⁸. In the present study, diarrhea was found as the most common gastrointestinal symptom in the patients, a finding in harmony with several reports of infection in India and Mexico^{37,38}.

Since the isolates of *H. nana* infecting humans and rodents are morphologically identical, the only way they can be reliably distinguished is by comparing the parasite in each host using molecular techniques¹⁷. Genetic diversity of *H. nana* has been studied using some genetic makers, such as cytochrome c oxidase subunit 1 (cox1) and the first and second internal transcribed spacer (ITS-1 and ITS-2) regions of nuclear ribosomal DNA (rDNA)^{39,40}. Furthermore, the utility of rDNA ITS2 in predicting the secondary structure from the primary sequence data can add ancillary information which could be further used in species identification⁴¹ as well as in providing resolution at higher taxonomic levels^{42,43}. These studies have indicated the existence of genetic variation of *H. nana* from different domestic and wildlife host species in different areas, suggesting that *H. nana* is a species complex, or “cryptic” species (morphologically identical but genetically distinct). In a study, the sequences of internal transcribed spacer 2 (ITS2) region of ribosomal DNA and a partial sequences of the mitochondrial cytochrome c

oxidase subunit 1 gene were compared between an isolate of *H. nana*, collected from a laboratory mouse (*Mus musculus*) from Japan, and a laboratory golden hamster (*Mesocricetus auratus*) from Uruguay. No sequence differences in the ITS2 were found between both isolates³⁹. In the current study, all ten positive specimens of *H. nana* successfully multiplied by molecular method and no sequence differences were found in the ITS2 between the isolates.

Conclusion

The PCR-sequencing technique used in the current study successfully and accurately identified all isolates. Thus, this technique could offer a valuable diagnostic tool when human eggs are the only source available to identify and diagnose *H. nana* parasite.

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Conflict of interest

We declare no conflict of interest.

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