Research Article *http://www.alliedacademies.org/parasitic-diseases-diagnosis-therapy/*
ISSN: 2591-7846

Molecular characterization of *Hymenolepsis nana* infecting human in Sohag, Egypt.

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Abstract

Background: *Hymenolepis nana* is the most common cestode in humans, rats and mice. It is not clear whether *H. nana* found in humans is the same species found in rodents or whether they are two distinct species morphologically identical yet genetically distinct.

Objective: To identify the genomic diversity of *H. nana* human isolates in Sohag, to determine their transmission patterns and to construct the phylogenetic tree to assess the evolutionary relationship of *H. nana* human and rodent isolates.

Methods: 20 *H. nana* positive stool samples were collected from outpatients' children referred to Sohag hospitals. Genomic DNA was extracted from stool samples. Mitochondrial cytochrome C Oxidase subunits 1 (*COX1*) gene of *H. nana* human isolates were amplified, sequenced and analyzed phylogenetically. The phylogenetic trees were reconstructed by neighbor joining and maximum likelihood methods based on *COX1* gene of *H. nana* human isolates obtained in this study and the known *H. nana* human and rodent isolates published in GenBank.

Results: All 20 *H. nana* isolates were successfully amplified and sequenced at the *COX1* and produced 5 different *COX1* gene sequences. The NJ and ML phylogenetic tree showed almost similar topology which suggested that *H. nana* human isolates of present study were closely related which means they came from the same origin, were more related to *H. nana* infecting human especially Latin America due to migration, differed notably from rodent isolates of previous studies which support that rodent to human is less likely to happen and were different from human isolate of Australia. This is explained by evolution in Australian isolates due early separated from rest of the world. Genetic differences were found 0.6% to 4% between human isolates of present study and rodent isolates of previous studies. The annotated sequences of our Egyptian isolates were deposited in GenBank of NCBI under the following accession number; MT093851, MT093852, MT093853, MT093854 and MT093855.

Conclusions: The Phylogeny of the *COX1* suggested that *H. nana* is a species complex or cryptic species (morphologically identical yet genetically distinct) and the life-cycle of *H. nana* that exists in Sohag is likely to Involve mainly human to human transmission.

Keywords: *H. nana*, mt *COX1*, PCR, Sequencing, Phylogeny

Accepted December 10, 2020

Introduction

Hymenolepis nana is the most prevalent parasite tapeworms in human [1]. It is capable of completing its life cycle without an intermediate host (direct life cycle) and it may also passage through an intermediate host [2]. This tapeworm is also found in the small intestine of rats and mice [3]. Rodents such as the black rat *Rattus rattus* and the house mouse *Mus musculus* are the most widespread mammals in the world. These rodents find stable environmental conditions and abundant food supplies in human settlements; in turns, this increase the probability of contact with residents when rodents invade household representing a potential health risk since *H. nana is* common parasite of rodents [4].

It is not clear whether *H. nana* found in humans is the same species found in rodents or whether they are two distinct species morphologically identical yet genetically distinct. Since its isolates infecting humans and rodents are identical, the only way they can be reliably distinguished is by comparing the parasite in each host using molecular techniques [5].

In addition to the results by [6] who demonstrated failure of development of *H. nana* human isolates in rodent hosts or to cysticercoid stage in the intermediate insect host which suggested that the life cycle of *H. nana* is mainly human to human transmission.

The same results were met with [7] in Mexico who reported intraspecific genetic differences within *H. nana* ranging from 0% to 5% in *COX1* gene which suggested presence of complex species within *H. nana* infecting humans and rodents.

Also, the control of a parasitic disease is dependent on the rapid and accurate detection of its causative agent, this necessitates traditional techniques being complemented by molecular tools that provide predictive data on genetic variation in and among parasites [8].

Mitochondrial coding regions such as the cytochrome C Oxidase subunit1 (*COX1*) gene have been successfully used to identify genetic variation between closely related parasite species or cryptic species [9].

Based on morphology, several studies have been conducted in Egypt to identify *H. nana* [10-12], however very few studies focused on the molecular and phylogenetic characterization of *H. nana* in Egypt. Therefore, there is a need to identify the genetic diversity of *H.nana* infecting human in Sohag.

Material and Method

Study area

This study was carried out in Sohag Governorate, Upper Egypt. Sohag is located in the southern part of the country toward 467 km to south of Cairo. It covers an extent of the Nile Valley with a total area of 1547 km^2, with estimated 5,338,025 people.

Sample collection and ethics statement

A total of 500 stool samples were collected from the outpatients aged 1 to 15 years, referred to medical laboratories from rural and urban areas in Sohag, Egypt during the period from November 2018 to June 2019. Informed consent was taken from the patients or children's guardians. The study was approved by Ethics Committee of the university. Diagnosis of *H. nana* was confirmed by direct wet-mount and formalin-ethyl acetate concentration methods.

Sample preparation and DNA extraction

20 positive stool samples of *H. nana* eggs were sieved and washed with distilled water by centrifugation for 10 min at 1,500 g. Processed samples were stored in -20°C until used for DNA extraction. Genomic DNA was extracted from approximately 180 mg-200 mg washed fecal pellets using QIAamp DNA Mini Stool Kit (Qiagen, Germany) according to the manufacturer's instructions. Eluted DNA was kept frozen at -20°C until its analysis with PCR [13].

PCR Amplification Protocol

A 391 bp DNA fragment of mt *COX1* gene of *H. nana* was amplified by PCR, using the following primer pairs previously described by [14]. The primers were provided by (Invitrogen, Thermo Fisher Scientific, USA) as following Table (1):

Table 1: *Primer of mt COX1 gene of H. nana.*

Primer name	Primer Sequence (5' to 3')
pra-Forward	TGGTTTTTTGTGCATCCTGAGGTTTA
prb-Reverse	AGAAAGAACGTAATGAAAATGAGCAAC

Each PCR mix was prepared in 50 µg total volume with 1 µ of template (50 ng), 10 pMoles of each primer, 45 µl of Ready Taq Mix Complete (Master Mix, Promega, USA), and nuclease free water (Qiagen, Germany) to complete the total volume of the reactions [15]. PCR reactions were performed in Thermal Cycler (Veriti, Applied Biosystems, USA**)** using the following cycling protocol as previously described by [5]: an initial denaturation at 94°C for 1 min 50 sec followed by 30 cycles each consisting of 94°C for 50 sec, 55°C for 1 min 30 sec and 72°C for 1 min 30 sec. Lastly final extension was at 72°C for 7 min. A negative control with absence of DNA was included in all PCR tests.

Agarose gel electrophoresis of PCR products

PCR products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) in 1x Tris-acetate-EDTA (TAE) buffer. A 100 bp DNA ladder was loaded in each gel then visualized under UV transilluminator. A negative control with absence of DNA was included on each agarose gel [16].

Sequence analysis

PCR products were purified by using a QIAquick PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. Purified PCR products were sequenced by Sangar method in both directions with PCR primers on an automated sequencer (the ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA), using a Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to manufacturer's instructions. The sequences obtained in the present study were aligned with available reference sequences from GenBank database using the Basic Local Alignment Search Tool (BLAST) [17]. Representative nucleotide sequences obtained in this study were deposited in the GenBank database.

Phylogenetic analyses

The phylogenetic trees were constructed using neighbor-joining (NJ) and maximum likelihood (ML) methods to determine the evolutionary relationship among the Egyptian *H. nana* human isolates in Sohag with GenBank reference sequences [18]. The trees were evaluated using the bootstrap test based on 1000 resamplings. The percentage of replicate trees or node reliability was shown next to the branches [19]. The trees were drawn to scale with branch lengths measured in number of nucleotide substitutions per site [20]. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA 7 (Molecular and Evolution Genetic Analysis version 7). The genetic differences in both datasets were estimated using p distance method [21].

Results

The prevalence of H. nana

Out of 500 stool samples, 29 (5.8%) isolates were contaminated with *H. nana* in formalin-ethyl acetate concentration. Of 29 patients, 15 were males and 14 were females. The prevalence of *H. nana* among males was 7.1% (15/213) where in females the prevalence was 4.8% (14/287).

PCR amplification of mt COX1 gene

PCR-amplification of mt *COX1* gene of 20 human stool samples yielded a single band of approximately 391 bp for *H. nana* on gel electrophoresis (Figure.1). The gels showed that the samples had different parasitic loads and this is this related to the concentration of *H. nana* infection in samples.

Sequence analysis

In the present study, 20 *H. nana* isolates were successfully

sequenced by amplification with primers pr-a and pr-b and showed that all PCR products had nucleotides belonging to *H. nana*. The *COX1* gene sequences obtained were the same in length (391 bp) among all specimens. 20 *H. nana* human isolates produced 5 *COX1* gene sequences with nucleotide differences between them. All 5 different sequences had the largest similarity with *H. nana* human isolates from Mexico (MN536021; [7]). Variation occurred in terms nucleotide substitutions (transition and transversion). Five nucleotide substitutions were shown at positions 71, 243, 264, 350. Two mutations from them were transition changes (T to C at 264 and G to A at 350) and three transversion changes (T to A at 71, A to T at 243 and G to T at 350).

Submission to NCBI

The annotated sequences of Egyptian *H. nana* human isolates were then deposited in the GenBank of NCBI under the following accession number; MT093851, MT093852, MT093853, MT093854 and MT093855. The results of homology analysis of *H. nana* isolates at the *COX1* loci (Table 2). Reference sequences used for phylogenetic analysis in this study with their isolates, hosts, sample types, localities and GenBank accession numbers of *COX1* sequences were illustrated in (Table 3).

Table 2: *Homology analysis in nucleotides at COX1 loci of H. nana.*

Isolate	No. of samples shared the same sequences (%)	Accession no$_a$	Accession no$_b$	Homology (%)	Nucleotide changes (Position)	Amino acid changes
Hn1	11samples (55%)	MT093851	MN536021	99.74%	T to C/(264)	Valine to Alanine
Hn2	4 samples (20%)	MT093852	MN536021	99.49%	A to T/(243) T to C/(264)	Tyrosine to Phenylalanine; Valine to Alanine
Hn3	2 samples (10%)	MT093853	MN536021	99.49%	T to A/(71) T to C/(264)	Phenylalanine to Isoleucine; Valine to Alanine
Hn4	2 samples (10%)	MT093854	MN536021	99.49%	T to C/(264) G to A/ (350)	Valine to Alanine; Alanine to Threonine
Hn5	1 sample (5%)	MT093855	MN536021	99.49%	T to C/(264) G to T/(350)	Valine to Alanine; Alanine to Serine

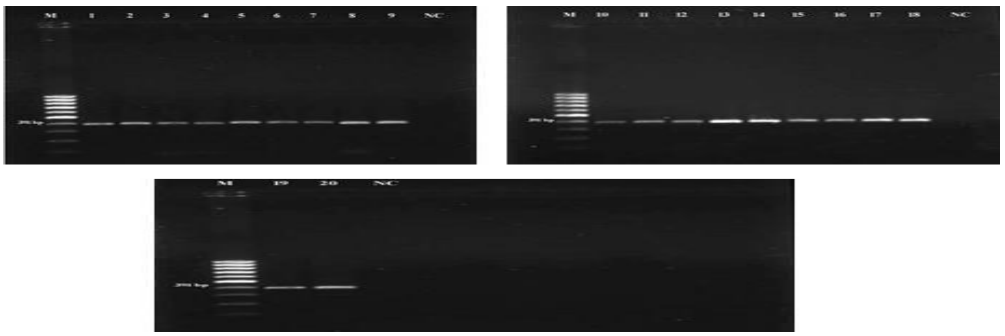

Figure. 1*: Agarose gel electrophoresis images that show the PCR product analysis of COX1 gene in H. nana human isolates. Where lane M: 100 bp molecular weight markers, lane (1-20) positive H. nana human isolates at 391bp PCR product and lane NC: negative control with no template DNA.*

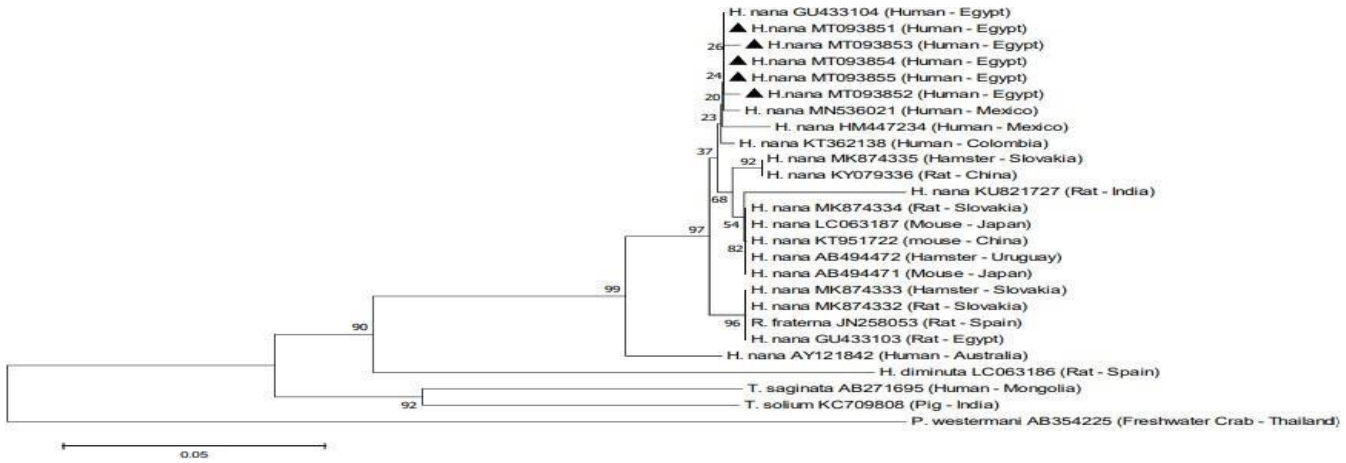

Figure 2: *Neighbor-joining tree of Egyptian H. nana human isolates based on nucleotide sequences of the COX1 gene. Phylogenetic analyses were conducted in MEGA7.Tree was inferred with p distance parameter model. H. nana human isolates were clarified in present study by (Δ). Each sequence obtained from GenBank was identified by the accession number, host origin and locality. Bootstrap values of 1000 replicates were shown at the nodes. The scale bar represented the estimated number of nucleotide substitutions per nucleotide site. P. westermani (AB354225) was selected as outgroup taxa.*

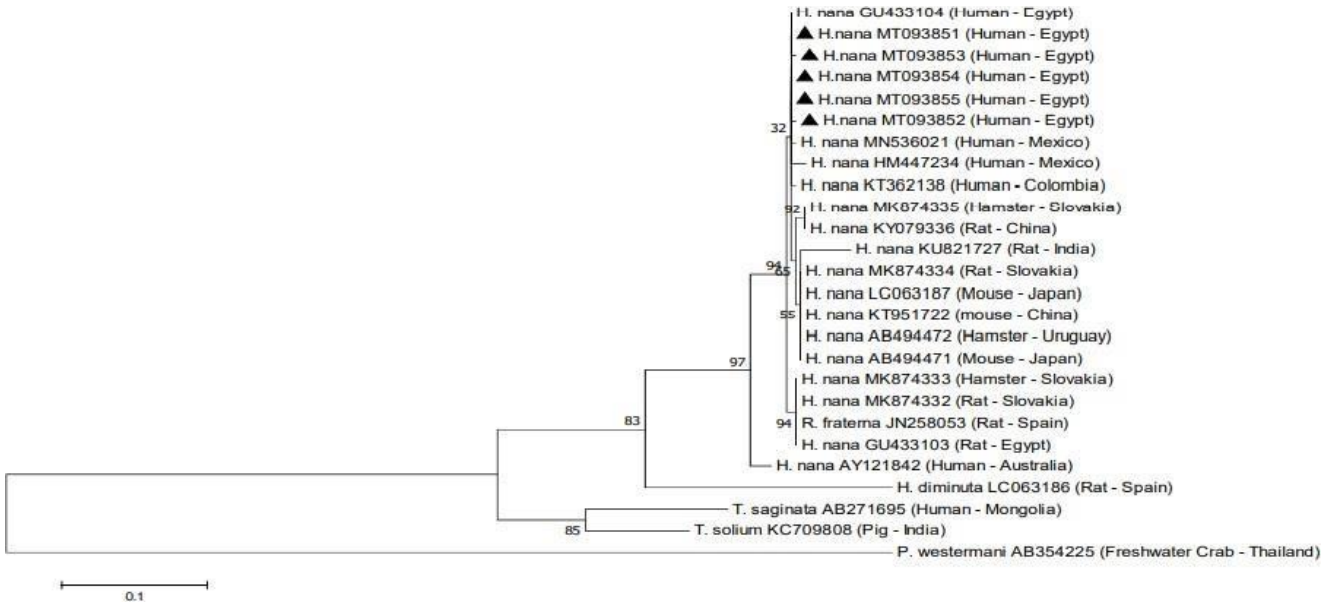

Figure 3: *Maximum likelihood tree of Egyptian H. nana human isolates based on nucleotide sequences of the COX1 gene. Phylogenetic analyses were conducted in MEGA7.Tree was inferred with the GTR+I+G substitutionmodel. H. nana human isolates were clarified in present study by (Δ). Each sequence obtained from GenBank was identified by the accession number, host origin and locality. Bootstrap values of 1000 replicates were shown at the nodes. The scale bar represented the estimated number of nucleotide substitutions per nucleotide site P. waterman (AB354225) was selected as outgroup taxa.*

- Accession nos [a]: indicating the novel sequences obtained in the present study
- Accession nos [b]: indicating the sequences downloaded from GenBank which have the largest homology with the sequences obtained in the present study

Phylogenetic analysis

Molecular phylogenetic trees were constructed using NJ and ML methods based on 26 *COX1* nucleotide sequences obtained in this study. NJ and ML trees were inferred with p distance and GTR+I+G substitution model respectively. *P. westermani* (AB354225) [22] was used as the outgroup. Bootstrap resampling of 1,000 replicates was done. Both NJ and ML analyses produced trees with the same topology and approximate relatively bootstrapped values (Figures 2 and 3). Phylogenetic analysis of the *COX1* nucleotide sequences revealed all *H. nana* human isolates of present study gathered with human and rodent isolates of previous studies in one clade with a high bootstrap support (99%) despite their host susceptibility differences. This clade was composed of three subclades of *H. nana* isolates.

All human-derived isolate from present study cluster together with human derived isolates from Egypt (Gu433104) [15], Colombia (KT362138) [23], Paraiso, Mexico (MN536021) [7], Sinaloa, Mexico (HM447234) in first subclade of *H. nana COX1* sequences with a low bootstrap support (23% in NJ tree or 20% in ML tree).

In addition, mouse isolate from Japan (AB494471) [14], hamster isolate from Uruguay (AB494472) [14], mouse isolate from China (KT9512722) [24], mouse isolate from Japan (LC063187) [25], rat isolates from India (KU821727) [26], brown rat isolate from China (KY079336) [13], rat and hamster isolates from pet rodents in Slovakia (MK874334, MK874335) [27] clustered together in second subclade of *H. nana COX1* sequences with moderate bootstrap support (68% in NJ tree or 65% in ML tree).

Lastly the rat isolate from Egypt (GU433103) [15], rat isolate from Spain (JN258053) [28], rat and hamster isolates from pet rodents in Slovakia (MK874332, MK874333) [27] cluster together in third subclade of *H. nana COX1* sequences with a high bootstrap support (96% in NJ tree or 94% in ML tree). The topology within these subclades suggested a division correlating with host origin, with isolates from the same host species clustering with each other however, this topology was not supported by bootstrap analysis. Pairwise distances between *H. nana* human and rodent isolates based on *COX1* nucleotide sequences were calculated by p distance model in MEGA 7. Intraspecific variations ranged from 0.6% to 4% between the human isolates of present study and rodent isolates of the previous studies.

DISCUSSION

H. nana (*Rodentolepis nana*) is the most common cestode in humans, rats and mice [29]. Some researchers described *H. nana* as being infective to both humans and rodents [30]. Others have concluded that the rodent form is a subspecies of *H. nana* var. fraterna, morphologically indistinguishable from the human form and only infective to rodents [31, 32]. It is not clear whether *H. nana* found in humans is the same species found in rodents or whether they are two distinct species morphologically identical yet genetically distinct [5]. Molecular prospecting is usually initiated when one suspects the presence of cryptic species or when one needs to be sure they are dealing with a single species

or population [33].

In the current study, the *COX1* gene sequences were the same in length (391 bp) among all specimens and 20 *H. nana* human isolates produced 5 different *COX1* gene sequences which had the largest similarity with *H. nana human* isolates from Mexico (MN536021) (99.74% with Hn1 and 99.49 % with Hn2, Hn3, Hn4 and Hn5). These results were very close with [14] in Japan who found that the two samples of *H. nana* isolated from distinct geographical areas, one from Uruguay and the other from Japan, were very close genetically. Also, it was reported that the genetic distances were not always related to geographical distances between the locations where each cestode had been collected [34, 35].

Nearly the same was reported by [33] who compared the genetic distances from pairs of congeners of platyhelminths for both mtDNA and ITS sequences and clearly demonstrated that mtDNA sequences accumulate nucleotide substitutions at a much higher rate than ITS. The higher rate of evolution and smaller effective population size of mtDNA sequences increased the probability of detecting diagnostic characters between cryptic species.

In the present study, the phylogenetic tree developed through the NJ and ML methods revealed almost similar topologies that all *H. nana* human and rodent isolates gathered in one clade with a high bootstrap support (99%) despite their host susceptibility differences. This clade was composed of three subclades.

First subclade consisted of human isolates from present study, Egypt (Gu433104) [15], Colombia (KT362138) [23], Paraiso, Mexico (MN536021) [7] and Sinaloa, Mexico (HM447234) with a low bootstrap support (23% in NJ tree or 20% in ML tree).

Second subclade consisted of mouse isolate from Japan (AB494471) [14], hamster isolate from Uruguay (AB494472) [14], mouse isolate from China (KT951722) [24], mouse isolate from Japan (LC063187) [25], rat isolate from India (KU821727) [26], rat isolate from China (KY079336) [13] and both rat and hamster isolates from Slovakia (MK874334, MK874335) [27] with moderate bootstrap support (68% in NJ tree or 65% in ML tree). Third subclade consisted of rat isolate from Egypt (GU433103) [15], rat isolate from Spain (JN258053) [28] and both rat and hamster isolates from Slovakia (MK874332, MK874333) [27] with a high bootstrap support (96% in NJ tree or 94% in ML tree).

The topology within the subclades suggests that *H. nana* human isolates of present study were closely related which means they came from the same origin, were more related to *H. nana* infecting human especially Latin America due to migration, differed notably from rodent isolates of previous studies which support that rodent to human is less likely to happen and were different from human isolate of Australia. This is explained by evolution in Australian isolates due early separated from rest of the world.

The results of the present study were supported by [5] in Australia who found that the isolates of *H. nana* in phylogenetic tree of mitochondrial *COX1* gene were divided into 2 clades, one containing the mouse isolates and the other containing the remaining human and mouse derived isolates. The topology within the latter clade suggests a division correlating with host origin, with isolates from the same host species clustering with each other. However, this topology was not supported by bootstrap analysis. In addition to the results by [6] who demonstrated failure of development of *H. nana* human isolates in rodent hosts or to cysticercoid stage in the intermediate insect host which suggested that the life cycle of *H. nana* is mainly human to human transmission.

The results did not differ from phylogenetics tree of *COX1 gene* constructed by [15] in Egypt, who demonstrated that both Egyptian murine isolates of hymenolipidid; *H. diminuta* and *H. nana*, were closer to each other than being to *H. nana* of human origin.

Also, the results were in accordance with phylogenetic tree of *COX1* gene of *H. nana* constructed by [27] in Slovakia in which rat and hamster isolates of *H. nana* from pet rodents in Kosice city of Slovakia (MK874332, MK874333) were located in a subclade, with a high bootstrap support (92%), together with rat isolate sampled from Egypt (GU433103) and a rat isolate from Spain (JN258053). Also rat and hamster isolates from pet shops in Persov city of Slovakia (MK874334, MK874335) clustered in a subclade with mouse isolate from China (KT951722), hamster isolate from Uruguay (AB494472) and brown rat isolate from China (KY079336).

Nearly the same phylogenetic analysis was reported by [7] who suggested that infection with *H. nana* was a non-zoonotic transmission based on *COX1* gene in children from rural Mexico. Besides they suggested that rodents were not the primary source of the *H. nana* infection for these children as they detected that the prevalence was 7.8% out of 135 children, but there were no eggs in stool specimens from 233 *Mus musculus* even in households where children were positive.

In the present study, Intraspecific genetic differences between the human isolates of present study and rodent isolates of the previous studies were varied from 0.6% to 4% in *COX*1 gene. These results agreed with [14] who observed that hymenolepidid species had larger intraspecific variations and smaller interspecific variations at the *COX1* locus than at the *ITS2* locus. Also, were close to those of [5] in Australia who found a genetic divergence of approximately 5% in the *COX1* gene between mouse and human isolates of *H. nana* however the *ITS1* and the nuclear gene paramyosin did not provide the level of heterogenicity between them. The same results were met with [7] in Mexico who reported intraspecific genetic differences within *H. nana* ranging from 0 to 5% in *COX1* gene which suggested presence of complex species within *H. nana* infecting humans and rodents.

Conclusion

Our study suggests that *H. nana* is a species complex or cryptic species and the life-cycle of *H. nana* exicted in the Sohag was likely to involve mainly' human to human' transmission through ingestion of fecal contamination. Our results indicate the further need to study the phylogeny of *H. nana* and characterizing the genetic differences within its population, not only at the individual gene level, but also at the whole genome level.

31. Schantz PM. Tapeworms (cestodiasis). Gastroenterol Clin North Am.1996; 25:637-53.

32. Lloyd S. Other cestode infections: Hymenolepiosis, Diphyllobothriosis, Coenurosis and other adult and larval cestodes. In: Zoonoses. Oxford Univer Press, London. 1998;651-63.

33. Vilas R, Criscione C, Blouin M. A comparison between mt DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of *platyhelminth* parasites. Parasitol. 2005;131(6):839-46.

34. Bowles J, McManus DP. Molecular variation in Echinococcus. Acta Trop. 1993;53:291-305.

35. Okamoto M, Bessho Y, Kamiya M, et al. Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the COX1 gene. Parasitol Res.1995;81(6):451-8.

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