Study on the genomic diversity of *Hymenolepis nana* between rat and mouse isolates by RAPD-PCR

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Summary

Hymenolepis nana is a common parasite of rodents as well as human intestine. This parasite has been reported from all over the world, including Iran. The infection rate has been reported up to 40% in some areas. The infection has various clinical manifestations. The parasite could establish severe hyperinfection in patients with immune deficiency. Regarding the rodents as hosts of the parasite, the infection may disseminate through these hosts to the nature. As *H. nana* is a zoonoses, phylogenic study of this parasite is of particular importance. Considering these criteria, the genomic diversity of 16 *H. nana* with the origin of Shiraz and Tehran were studied among the worms of mice and rats by RAPD-PCR. Genomic DNA extracted from individual worms by proteinase K method and three oligonucleotides primer (AB1-17, UBC-358, UBC-387) were used for RAPD-PCR. Similarity index were calculated by Nei and Li method. Data were analysed using UPGMA analysis and dendrograms were obtained by group average method with 100 bootstrapping analysis. The range of genomic similarity determined among specimens by AB1-17 primer was 48.3-90%, by UBC-358 primer 55-87% and by UBC-387 primer 53-97%. Regarding our data and genomic similarity indexes, various isolates were found in both specimens of rats and mice. However no differences were obtained between *H. nana* from rat or mouse isolates by these primers. The results showed that it is not possible to divide the isolates into two distinct groups based on their origin as Tehran and Shiraz.

Key words: *Hymenolepis nana*, RAPD-PCR, Mouse, Rat, Genomic diversity

Introduction

Hymenolepis nana is the common tapeworm of humans throughout the world (Marquart *et al*., 2000). It is more common in warm climates (Beaver *et al*., 1984). This tapeworm is found in the small intestine of rats, mice and humans (Marquart *et al*., 2000).

The prevalence of *H. nana* is variable in the world. In Iran the most prevalent area of human hymenolepiasis is Khouzestan with up to 31.8% infection rate (Farahnak, 2001). The infection rate of *H. nana* in rodents of Khouzestan has been reported 31.3% (Sadjjadi and Massoud, 1999).

H. nana species in different rodents (rats, mice, etc) are morphologically identical to human *H. nana* (Ferretti *et al*., 1981). Hence, a possible identity between two species is important epidemiologically (Ferretti *et al*., 1981).

Despite revised nomenclature, the original confusion of speciation and host specificity remains to be solved (Macnish *et al*., 2002). It is necessary to examine the endemic strains to express a through opinion.

In this regard, biological, taxonomic and epidemiological studies on *H. nana* in various hosts could be helpful. The genomic characteristics of *H. nana* isolates in different hosts will also help us in determining host specificity and transmission patterns. This will also allow a more appropriate approach to control the

infection in endemic communities (Macnish *et al*., 2002).

To date, the molecular studies on *H. nana* isolates are rare (Okamoto *et al*., 1997; Macnish *et al*., 2002).

A DNA polymorphism assay based on random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used in this study. It was based on the amplification of a random DNA segment with a single primer of arbitrary nucleotide sequence using polymerase chain reaction (Williams *et al*., 1993). This method is very rapid, simple and generates a reproducible fingerprint of the PCR product. In addition, it does not depend on previous knowledge or availability of the DNA sequence of the target, and it does not generally require DNA hybridization. The potential use of RAPD-PCR in taxonomy and population genetics has been widely documented (Morgan and Blair, 1995). RAPD-PCR which has been used for phylogenetic studies in different parasites is a strong tool in hands of parasitologists for gene mapping and phylogenetic studies. The aim of the present study is to find the genomic diversity between rat and mouse isolates of *H. nana* originated from different localities.

Materials and Methods

Collection of parasite

A total of 30 adult worms of *H. nana* from small intestine of laboratory mice originated from Laboratory Animal Research Center at Shiraz University of Medical Sciences were collected. The specimens were washed repeatedly in PBS followed by 70% ethyl alcohol and stored at -20°C until use.

A total of six *H. nana* collected from intestine of rats received from School of Public Health at Tehran University of Medical Sciences were also used for the study.

Preparation and extraction of DNA

From each individual worm 2-5 mm were cutted, crushed and digested in 300 µl lysis buffer (tris-Cl, 10 mM, pH = 8; ethylen dinitrilo tetra-acetic acid (EDTA), 1 mM; Tween 20, 0.2%) (Merck, Germany) in the presence of 10 µl proteinase K (20 mg/ml) (Boehringer Mannheim, Germany) at 37°C for 24 hrs for mouse specimens and >5 days for rat specimens while stirring. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) as described before (Sambrook *et al*., 1989). The DNA was precipitated with cold ethanol (-20°C) followed by twice washing in 70% ethanol. The DNA was dried at 37°C and the precipitate was resuspended in 50 µl deionized distilled water and then stored at -20°C until used.

PCR reaction

Twenty primers each consisted of 10-mers were tested. A total of 3 primers were selected based on the production of distinct and intense bands. These primers were as follows: AB1-17: 5'AGGGAACGAG 3'; UBC-358: 5'GGTCAGGCCC 3' and UBC-387: 5'CGCTGTCGCC 3' (Toobanegin, Iran).

Total DNA was used as template for amplification of DNA fragments by the PCR.

Each reaction, in a total volume of 25 µl contained 1 X PCR buffer (20 mM tris-HCl, pH = 8.4; 50 mM KCl), 0.25 mM of mixed deoxynucleotide triphosphatase (dNTPs), 2.5 mM $MgCl_2$, 1.5 units Taq DNA polymerase (Sinagen, Iran), 20 pM of each primer, ~ 40 ng genomic DNA, over laid with 30 µl mineral oil (Merck, Germany).

The DNA was initially denatured for 5 min at 94°C. The PCR cycles were carried out for 1 min at 94°C, 1 min at 35°C and 2 min at 72°C. A total of 35 cycles followed by 1 extensive polymerization reaction (5 min at 72°C). The PCR was performed in a progen thermocycler (Techene Cambridge, England). Controls (without DNA) always tested as negatives.

Agarose gel electrophoresis of RAPD products

Ten microliters of amplified reaction was mixed with 2 µl of loading buffer (3 mg bromophenol blue; glycerol 80%; 70 ml of 0.5 M EDTA, pH = 8) (Merck, Germany) and electrophoresed on a 1.5% agarose gel (Boehringer, Germany) in TAE buffer as described (Sambrook *et al*., 1989) and visualized by ethidium bromide staining

(Merck, Germany). For each primer used, a tube containing all the components except the template DNA was included as a control to detect the potential contamination. In addition, a molecular weight marker (Gene Ruler^{TM} 100 bp DNA ladder Plus) (Fermentas, Lithuania) was used for the study.

Data analysis

RAPD profiles were used to measure genetic similarity among the worms. Each DNA band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 for presence or absence, respectively. The profiles were analysed by Nei and Li method: GDxy = 1-2 Nxy/Nx+Ny; Nx = number of bands in x isolate, Ny = number of bands in y isolate and Nxy = number of bands in both x and y isolates. Similarity matrix was computed and genomic similarity was summarized in the form of a dendrogram treelike structure with UPGMA (unweighted pair group method with arithmetic averages) with 100 bootstrapping analysis (Van de Peer and De Wachter, 1997)

Results

The results of RAPD-PCR on mice and rat proglotids from different geographical areas (S isolates are the mouse isolates, obtained from Shiraz and T isolates are the rat isolates from Tehran) are shown as follows:

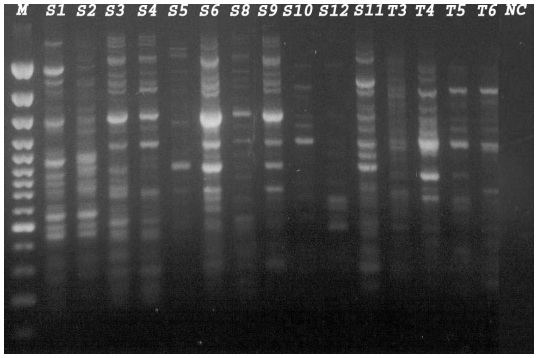

Fig. 1: Agarose gel electrophoresis (1.5%) of RAPD-PCR products in total genomic DNA of mouse and rat proglotids of *H. nana* by AB1-17 primer

RAPD-PCR by AB1-17 primer

The mean ± SD of bands obtained by AB1-17 primer was 19.5 ± 3.2. The range of the bands was between 13 to 23. Fig. 1 shows the electrophoresis of RAPD-PCR products in total genomic DNA from 9specimens of mouse and 4 specimens of rat proglotids. Regarding Fig. 2 as phylogenic tree, it is shown that the isolates in this dendrogram are divided into two clusters: cluster one containing T5 and T6 isolates and cluster two containing S3, S4, S1, S2, S5, T3, S9, S11, S6, S8 and T4. Maximum similarity is seen between S_1 and S_2 in cluster two (90%) and minimum similarity is seen between T5 and T6 and other isolates (48.3%). However rat T3 isolate in cluster two showed genomic similarity between rat and mouse isolates.

RAPD-PCR by UBC-358 primer

Fig. 3 shows the electrophoresis of RAPD-PCR products in total genomic DNA of 10 mice and 5 rats proglotids. The mean ± SD of bands obtained by UBC-358 primer was 16.9 ± 2. The range of the bands was between 14 to 21. In Fig. 4 as phylogenic tree, it is shown that the isolates in this dendrogram are divided into two clusters: cluster one containing S3, S4, S5, S1, S2, T4, T5 and T6 and cluster two containing S12, T3, S6, S8, T2, S10 and S11. Maximum similarity (87%) is seen between T5 and T6 isolates of rat. The isolates could not be divided into two distinct isolates in rat and mouse.

RAPD-PCR by UBC-387 primer

Fig. 5 shows the electrophoresis of RAPD-PCR products in total genomic DNA of 6 mice and 3 rats proglotids. The mean ± SD of bands obtained by UBC-387 primer was 17.2 ± 2.7. The range of the band numbers was between 15 to 24. The phylogenic tree is shown in Fig. 6. The isolates in this dendrogram are divided into two clusters: cluster one containing T6, T5, S10 and T2 and cluster two containing S8, S11, S6, S4 and S3. Maximum similarity (97%) is seen between S3 and S4 and cluster two containing mouse isolates.

RAPD-PCR by three primers (AB1–17, UBC-387 and UBC-358)

The results of analysis of RAPD-PCR of 11 mice and 5 rats proglotids of *H. nana*

using three primers are shown in Table 1 and Fig. 7. As shown in the dendrogram the isolates in this tree are divided into two clusters: cluster one containing T2, S5, T4, T6 and T5 and cluster two containing S12, T3, S11, S10, S8, S6. S2, S1, S3 and S4. Maximum similarity (84%) is seen between S4 and S3. However, there is not obvious

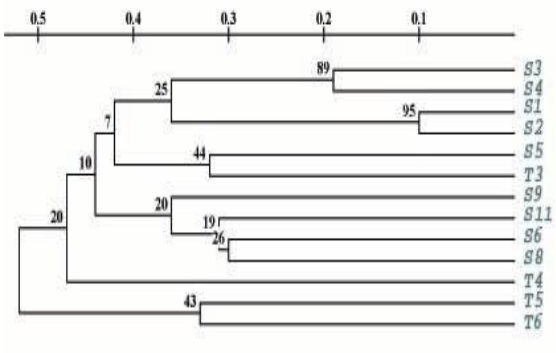

Fig. 2: Dendrogram resulting from analysis of RAPD-PCR of rat and mouse proglotids of *H. nana* using AB1-17 primer and UPGMA analysis with 100 bootstrapping

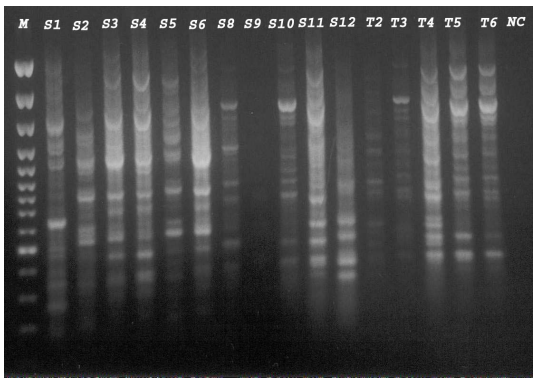

Fig. 3: Agarose gel electrophoresis (1.5%) of RAPD-PCR products in total genomic DNA of mouse and rat proglotids of *H. nana* by UBC-358 primer

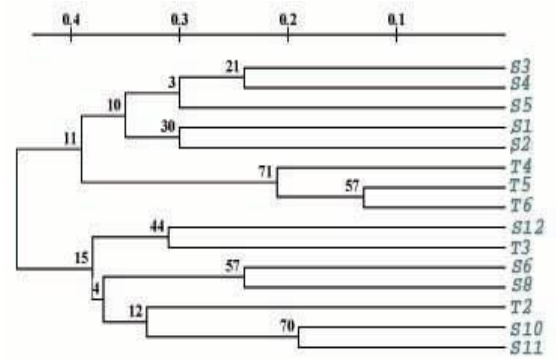

Fig. 4: Dendrogram resulting from analysis of RAPD-PCR of rat and mouse proglotids of *H. nana* using UBC-358 primer and UPGMA analysis with 100 bootstrapping

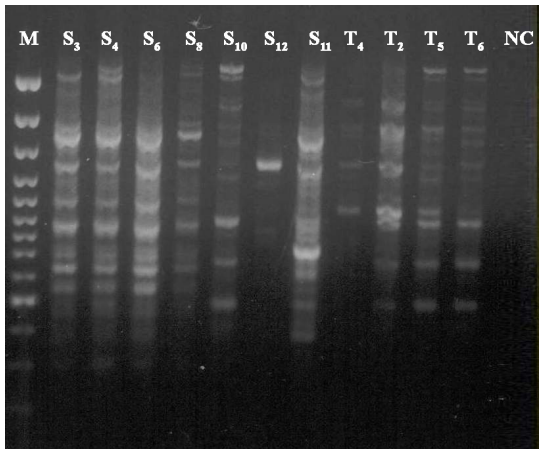

Fig. 5: Agarose gel electrophoresis (1.5%) of RAPD-PCR products in total genomic DNA of mouse and rat proglotids of *H. nana* by UBC-387

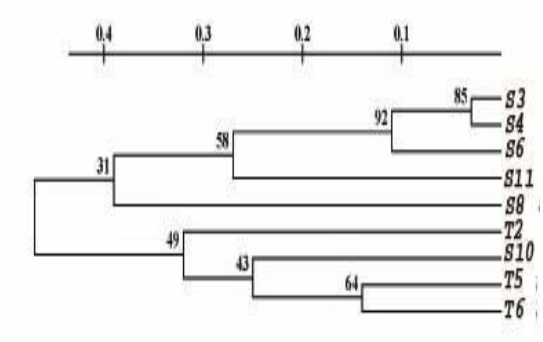

Fig. 6: Dendrogram resulting from analysis of RAPD-PCR of rat and mouse proglotids of *H. nana* using UBC-387 primer and UPGMA analysis with 100 bootstrapping

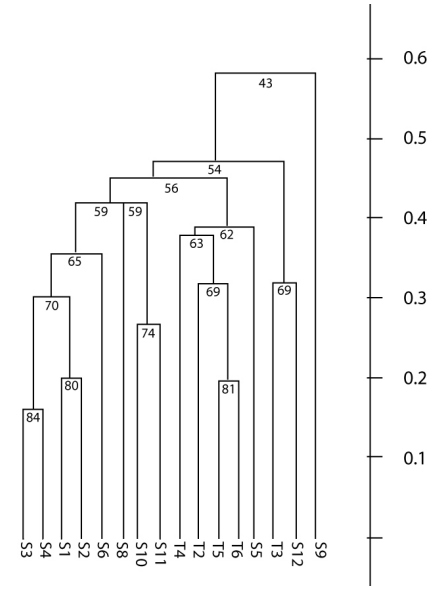

Fig. 7: Dendrogram resulting from analysis of RAPD-PCR of rat and mouse proglotids of *H. nana* using three primers and UPGMA analysis with 100 bootstrapping

Table 1: Matrices resulting from analysis of RAPD-PCR of rat and mouse proglotids of *H. nana* using three primers

	S_1	S_2	S_3	S_4	S_5	S_6	S_8	S_9	S_{10}	S_{11}	S_{12}	T_2	T_3	T_4	T_5	T_6
S_1	0															
S_2	0.8	0														
S_3	0.68	0.68	0													
S_4	0.61	0.69	0.84	0												
S_5	0.63	0.61	0.60	0.63	0											
S_6	0.59	0.60	0.70	0.70	0.57	0										
S_8	0.54	0.53	0.62	0.59	0.54	0.69	0									
S_9	0.53	0.52	0.49	0.48	0.59	0.68	0.67	0								
S_{10}	0.42	0.44	0.58	0.58	0.59	0.63	0.65	0	0							
S_{11}	0.56	0.59	0.69	0.64	0.59	0.65	0.59	0.60	0.74	0						
S_{12}	0.46	0.48	0.42	0.50	0.58	0.56	0.60	0	0.65	62	0					
T_2	0.57	0.48	0.56	0.52	0.65	0.51	0.59	0	0.56	0.68	0.64	0				
T_3	0.59	0.48	0.55	0.58	0.65	0.61	0.62	0.59	0.63	0.64	0.69	0.62	0			
T_4	0.56	0.66	0.61	0.56	0.58	0.53	0.55	0.43	0.51	0.63	0.50	0.63	0.51	0		
T_5	0.44	0.55	0.46	0.51	0.61	0.44	0.45	0.42	0.63	0.54	0.48	0.70	0.44	0.53	0	
T_6	0.57	0.64	0.53	0.53	0.62	0.54	0.50	0.49	0.64	0.60	0.58	0.68	0.52	0.72	0.81	0

differences between the two clusters, as both mouse and rat isolates are seen in both clusters.

Discussion

RAPD-PCR has been used for several phylogenic studies. The validity of this test has been approved for genomic differences in different studies. (Motazedian *et al*., 1996; Intapan *et al*., 2004; Zemanova *et al*., 2004).

In this study a total of 16 *Hymenolepis* specimens: 11 from mouse and 5 from rat were compared by molecular method. The result obtained by AB1-17 primer showed two clusters in which the composition did not correlate with host (mouse or rat). By UBC-358 and UBC-387 primers also two clusters were observed in which both mouse and rat specimens are scattered in each cluster. Variation was found between *H. nana* of both isolates of rat and mouse. In fact all isolates showed a pattern of bands different from each other. Thus RAPD analysis allowed all isolates to be differentiated.

The results of our research is in accordance with Hossain and Jones (1963) and Monaloy (1972) who showed that the chromosome number of *H. nana fraterna*, irrespectively from mice or rats were 2n = 12. Another study showed that karyotyping studies are not sufficient for showing the differences between the chromosomes of the parasites at the level of strains (Ardalan and Dalimi, 1996). They suggested the DNA studies for this item. In another study no sequence differences were found by the results of ITS2 sequences analysis between the isolates of Japan and Uruguay (Okamoto *et al*., 1997).

A molecular phylogeny of nuclear and mitochondrial sequences in *H. nana* carried out in Australia (Macnish *et al*., 2002). In this study, isolates of *H. nana* from rodent and human hosts from a broad geographical range were sequenced at the ribosomal first internal transcribed spacer (ITS1), the mitochondrial cytochrome C oxidase subunit 1 (CO1) gene and the nuclear paramyosin gene loci. Twenty three isolates of *H. nana* were sequenced at the ITS1 locus confirming the existence of spacers which, although were similar in length (approximately 646 bp), differed in their primary sequences. This led to the separation of the isolates into two clusters when analysed phylogenetically. This sequence variation was not, however, related to the host of origin of the isolates, and thus was not a marker of genetic distinction between *H. nana* from rodents and humans. Sequencing of a 444 bp fragment of the CO1 gene of 9 isolates of *H. nana* from rodents and 6 from humans identified a phylogenetically supported genetic divergence of approximately 5% between some mouse and human isolates. They suggested that *H. nana* is a complex species or cryptic species (morphologically identical yet genetically distinct). A small segment of the nuclear gene paramyosin (625 bp or 840 bp) was sequenced in 4 mice and 3 human isolates of *H. nana*. However, this gene did not provide the level of heterogenicity

required to distinguish between isolates from rodent and human hosts (Macnish *et al*., 2002).

It was reported that in the case of cestodes which parasitize the domesticated animals, genetic distance were not always related to geographical distances between the locations where each cestode had been collected (Bowles and McManus, 1993; Okamoto *et al*., 1995). A very low (99.5 - 100%) intra specific variation has been detected in mouse isolates from Australia, Japan and Italy. However, extensive intra specific variation was found between two Portuguese mouse isolates (Macnish *et al*., 2002).

Currently the yardstick for delineating species strains on the basis of genetic differences is unresolved in literature and remains contentious (Morgan and Blair, 1995; Haag *et al*., 1997; Blouin *et al*., 1998; Sorensen *et al*., 1998). Although some studies have proposed that if within species variation at particular genetic loci is low this supports the existence of a species (Hung *et al*., 1999). Others suggest that caution is required in interpreting the genetic data in the absence of any supportive biological data (Thompson and Lymbery, 1990; Blouin *et al*., 1998; Sorensen *et al*., 1998; Thompson *et al*., 1998; Tibayrenc, 1998, Macnish *et al*., 2002). Regarding our data and genomic similarity indexes, various isolates were found in both specimens from rats and mice. However no differences were obtained between *H. nana* from rat or mouse isolates. The results also showed that it is not possible to divide the isolates into two distinct groups based on their origin as Tehran and Shiraz.

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