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Original Article

A New Primer Pair in ITS1 Region for Molecular Studies on *Echinococcus granulosus*

HR Rahimi [1], *EB Kia [1], SH Mirhendi [1], A Talebi [2], M Fasihi Harandi [3], N Jalali-zand [1], MB Rokni [1]

[1]Dept. of Medical Parasitology & Mycology, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran
[2]Al-Zahra Hospital, Isfahan University of Medical Sciences, Iran
[3]Dept. of Medical Parasitology & Mycology, Faculty of Medicine, Kerman University of Medical Sciences, Iran

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Abstract

Background: *Echinocuccus granulosus*, the causative agent of cystic echinococcosis has long been recognized as having a high degree of genetic divergence. The strains characterization seems to be essential for the establishment of a preventive and control strategy in every endemic area. Using DNA based methods for strain /genotype characterizations of *E. granulosus* have some difficulties, especially access to an efficient and pure concentration of DNA and proper primers. **Methods:** Using grinder method, a pure and high concentration DNA was extracted from 10 human hydatid cysts collected from Isfahan (central Iran) hospitals, and processed for PCR reaction. **Results:** Using DNASIS, the primers were designed in internal transcribed spacer 1 (ITS1) region, following analysis of 30 *E. granulosus* nucleotide sequences, extracted from gene bank. **Conclusion:** This new and specific *E. granulosus* primer which amplified DNA thoroughly can be applied for molecular studies on echinococcosis.

Keywords: Echinococcus granulosus, *PCR, Primer design, DNA, ITS1, DNASIS*

Introduction

Hydatidosis caused by dog small tapeworm, *Echinococcus granulosus*, is known to be one of the most important parasitic zoonoses all around the world. Adult worms live in the small intestine of canids, mainly dogs. Hydatid cyst develops in the internal organs of human and herbivore intermediate hosts, mainly in the liver and lungs. It has long been recognized that *E. granulosus* possesses a high degree of genetic diversity. Various strains (designated G1 to G10) exhibit differences in morphology, development rate, host range, pathogenicity and geographical distribution (1). More research is required to determine the host and geographic ranges of these strains and whether genetic characteristics are conserved between different endemic regions (2). Although use of morphology is the most rapid, classical and practical method for study on parasite diversity, however, it is not efficient and valid for study on intraspecific variations. Moreover, some parasites with similar morphology show epidemiological differences (3). Therefore, in order to distinct strain/genotype differences within *E. granulosus*, DNA based methods are required (4). However, these methods sometimes, have some difficulties like the lack of access to amplicons with efficient and pure concentration of DNA obtained by PCR. The most important reason for this contradiction is the use of *Schistosoma mansoni* sequence for primer designing as followed in some molecular studies on *E. granulosus* (5).

In the current study, following consideration of different sequences of *E. granulosus* in gene bank, a new and specific *E. granulosus* primer was designed and applied thoroughly in PCR reaction.

***Corresponding author:** Tel/Fax: +98 21 88951392, E-mail: eshratkia@yahoo.com

Materials and Methods

Samples Human hydatid cysts were collected from 10 patients who had operations in hospitals affiliated to Isfahan University of Medical Sciences, Iran. The contents of every cyst, soon after drainage, were transported to the laboratory, kept in normal saline container. Then the samples were divided in several sterile tubes, washed a few times until the supernatant looked clear. The sediments of each tube were microscopically examined for the presence of protoscolices. Afterwards, the sediments containing protoscolices were removed to sterile screw capped containers with a few drops of 70% ethanol alcohol, keeping at 4 °C.

DNA extraction Protoscolices were washed three times with lysing buffer (NaCl 5M, EDTA 0.5, TrisHcl 1 M). Then, after adding 250 µl lysing buffer, they were shattered using mechanical grinder. Afterwards, lysing buffer and SDS in equal volume were added and mixed prior to addition of 10 µl protease K (100 mg/ml), placing in vortex and then incubating at 56 °C overnight. Next day, 500 µl phenol cholorophorm isoamil alcohol was added and spun at 5000 g for 5 min. The supernatant, then, was removed, and after chloroform addition and spinning, 50 µl sodium acetate (3 M, pH= 5.2) and 1000 µl ethanol were added and left at -20 °C freezer for half and hour. Thereafter, it was spun at 1200 g for 15 min and the sediment was washed by 70% ethanol. Later, the pellet was dissolved in 50 µl sterile super double distilled water, leaving at -20 °C for PCR process.

Primer design Nucleotid sequences of 30 *E. granulosus* isolates were extracted from gene bank through internet. Using DNASIS software, these nucleotides sequences were analyzed and ultimately two forward and reverse primers selected. After considering the coordination of the selected primers, lack of dimer primer and the capability of the primers in replication of desired fragments, the synthesis of the designed primers was ordered.

The characterization of the 30 *E. granulosus* isolates is presented in Table 1.

Table 1: The characterization of 30 *E. granulosus* isolates

Row No.	Strains	Nucleotides No.	Accession No.	Row No.	Strains	Nucleotides No.	Accession No.
1	EG 13.2	953	AJ 245930	16	33	965	AJ 237826
2	EG 13.2	953	AJ 245929	17	33	975	AJ237825
3	EG13.2	957	AJ 245928	18	33	977	AJ237824
4	174	979	AJ 237838	19	33	952	AJ237823
5	174	984	AJ 237837	20	33	976	AJ237822
6	7	986	AJ 257836	21	G8	984	AJ237821
7	7	840	AJ 237835	22	G8	974	AJ237784
8	44	990	AJ 237834	23	G8	982	AJ237783
9	41	964	AJ 237833	24	G8	965	AJ237782
10	41	970	AJ 237832	25	G8	957	AJ237781
11	33	967	AJ 237831	26	G1	904	AJ237777
12	33	936	AJ 237830	27	G6	964	AJ237776
13	33	990	AJ 237829	28	G6	1045	AJ237775
14	33	996	AJ 237828	29	G5	1024	AJ237774
15	33	986	AJ 237827	30	G5	1023	AJ237773

PCR In the current study nested PCR was applied for amplifications of the desired fragment. In first step PCR, the primers introduced by other authors (5) were used as outer primers for amplifications of an 1800 bp fragment. These primers were as follows:

EGF 1 (5 CCA AAC TTG ATC ATT TAG AGG AAG 3)

EGR 2 (5 TAT GGG CCA AAT TCA CTC ATT ACC 3)

The thermal profile, at first step PCR, was as follows: one 95 ºC cycle for 6 min for early denaturation, then 94 ºC for 45s (denaturation), 55 ºC for 1 min and 30 s (annealing) and 72 ºC for 2 min (extension). All three steps were repeated for 30 cycles and for the final extension 72 ºC for 5 min was applied. In the second PCR, the primers designed for the current study were used as internal primers for amplification of a 1000 bp fragment. The thermal profile in this step was as follows:

One cycle of 95 ºC for 2 min (primary denaturation), 94 ºC for 30 s, 55 ºC for 45 s, and 72 ºC for 1 min, all steps repeating for 30 cycles. For final extension 72 ºC for 5 min was used. The internal primers were as follows:

EgF: (5 GTC GTA ACA AGG TTT CCG TAG G 3)

EgR: (5 TAG ATG CGT TCG AAG TGT CG 3)

The concentration of the materials for both steps of PCR were similar including PCR buffer 10 times dilution, MgCl2 2 mM, dntp 0.1 mM, forward and reverse primers (each 25 pm), Taq polymerase 0.5 U.

Electherophoresis For visualization of the ex-tracted DNA and PCR products, electherophoresis was performed by adding 7 µl of the sample to 1.5% gel agarose containing 0.5 Mg/ml ethidium bromide stain for 45 min at 100 V. The bands were observed by UV light and photos prepared digitally.

Results

In this study, 10 human hydatid cysts belonged to 6 male and 4 female patients, ranging from 12 to 68 years old, were collected during their operations for hydatidosis in Isfahan hospitals and processed for extraction of *E. granulosus* DNA. Using grinder method, DNA from all 10 samples was extracted successfully. Fig.1 (a) represents the electrophoresis results of extracted DNA of all hydatid cysts samples. According to this figure, for all samples there was at least one heavy band correspondence to extracted genomic DNA. Fig. 1(b) illustrates the first step of PCR results, using outer primer. In all samples a fragment, about 1800 bp, was observed, which was not sharp enough. Therefore, nested PCR was performed, using inner primers. Fig. 1 (c) shows the correspondence results. Accordingly, in this step the extracted genomic DNA has been much amplified, thus, the concentrated and sharp bands are clearly visible.

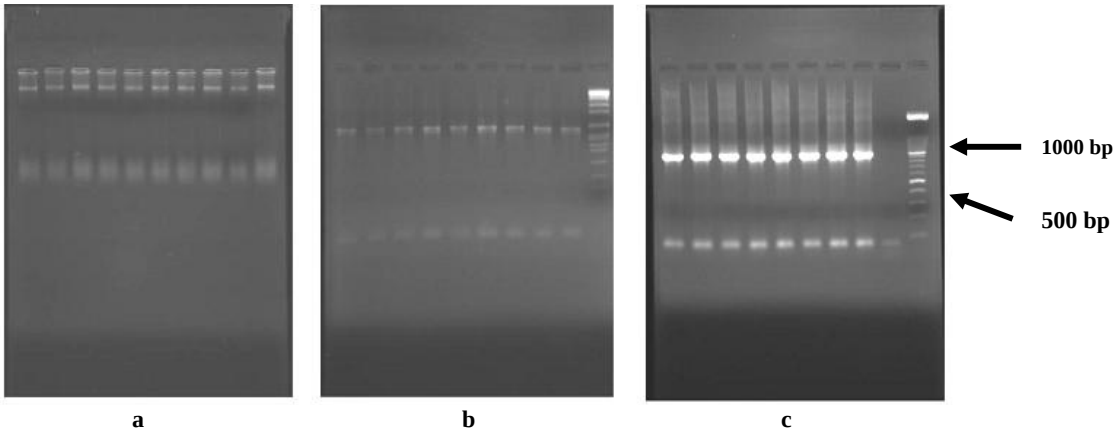

Fig. 1: a. Electerophoresis results of extracted DNA of 10 hydatid cysts samples; b. results of first step PCR using outer primer; c. results of nested PCR using inner primers.

Discussion

The taxonomy of *Echinococcus* has suffered from many decades of uncertainty regarding the taxonomic status of described species and subspecies (6). There is now considerable evidence demonstrating the importance of determining the nature and extent of strain genotypic variation in *Echinococcus* (1, 7). The use of morphology, solely, is not efficient for determination of genetic diversity. Recently, DNA characterization procedures have been used for study of *Echinococcus* variations, as with a few studies in Iran. First of them, was a preliminary study, based on mitochondrial DNA markers, on 16 isolates of human and domestic animals from different areas of the country, indicating the presence of two distinct sheep strain and camel strain in Iran (8). Thereafter, in a study, using both PCR-RFLP of ITS1 and morphological criteria, the sheep strain was the most common genotype in Iran, and camel genotype was found infecting 3 out of 33 human cases (5). Although DNA based methods, has advantage of their usefulness in the taxonomy at the level of genus, species and subspecies, nevertheless, application of molecular methods often needs specific attention to utility of proper primers and access to pure and efficient amount of DNA. Researchers usually encounter difficulties while using PCR-RFLP in characterization of *E. granulosus* isolates (9). This is probably as a result of DNA extraction procedure, or/ and using non-specific primer, as those derived from *Schistosoma mansoni*. The sequences of primers used by some other authors (8) are as follows:

Outer primers:
egf1: cca aac ttg atc att tag agg aag
egr2: tat ggg cca aat tca ctc att acc
Inner primers:
bd1: gtc gta aca agg ttt ccg ta
4s: tct aga tgc gtt cga agt gtc gat g

In the present study, two attempts were tried to overcome above mentioned troublesome: First, designing of a new primer in ITS1 region based on certain sequences of *E. granulosus;* second, improving DNA extraction procedures. According to the results, in both attempts successful outcomes were obtained:

-DNA concentration was high enough to perform any PCR technique.
-The designed primer pair was able to amplify DNA thoroughly.

The products of this PCR are required for further post PCR efforts such as PCR-RFLP, for species and strains differentiations, and also for sequencing determination, both requiring a high concentration of PCR product, and free of extra bands. The PCR product of nested PCR obtained in the current study was properly compatible with such necessary requirements.

Conclusively, in the future, using the designed primer and sequencing the PCR products, a proper RFLP can be introduced for molecular studies on *E. granulosus* to characterize a high number of *E. granulosus* from human and different animal origins and from different geographical areas.

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