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Role of *Echinococcus granulosus* Mitochondrion Partial Gene Polymorphism in Responsiveness to Albendazole Treatment in Experimentally Infected Mice

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Abstract

Background and objective: Cystic echinococcosis which caused by the larval stage of the tapeworm *Echinococcus granulosus* is one of the important public health challenges worldwide. Various strains have been recorded. Available evidences revealed that pathogenesis of the disease has been associated with parasite genotype. The current study aimed to investigate role of mitochondrion partial genome polymorphisms, in responsiveness to albendazole treatment in murine model.

Methods: Hydatid cysts were obtained from infected livers of sheep. Protoscoleces from each isolate were separated into two batches. First preserved in ethanol (70%) at -20 ^{o} C for molecular investigation. The second part was used for direct *in vivo* experiment. Genomic DNA was extracted and mitochondrion partial genome was amplified and sequenced. Fourteen groups of Mice (twelve mice each) were infected experimentally with protoscoleces from 14 hydatid cysts isolates. Responsiveness of each isolate to albendazole was assessed.

Results: The sequencing profiles revealed no polymorphisms in 6 of the 14 studied isolates, while minor sequence polymorphisms, 0.33 %, that resulted in corresponding amino acid replacement, were observed in 3 of the isolates. Furthermore, remarkable DNA sequence polymorphisms were observed in 5 of the isolates, that caused affectable amino acid replacement in the suggested polypeptide sequences. Treatment efficacy of albendazole was positively correlated (r = 0.128) (*P* <0.001) with the polymorphism in *E. granulosus* mitochondrion partial genome.

Conclusion: A polymorphic sequence profiles of *E. granulosus* mitochondrion partial genome can be used to detect the sensitivity of the causative strains to albendazole. However, further studies should be conducted to involve hydatid cyst isolates from other intermediate hosts, and to assess the effectiveness of other anthelmintic drugs against various strains of the tapeworm *E. granulosus*.

Keywords: *Echinococcus granulosus*; Hydatid cyst; Albendazole; Gene polymorphism; Mitochondrion partial genome.

Introduction

Cystic echinococcosis which caused by the larval stage of the tapeworm *Echinococcus granulosus* (*E. granulosus*) is one of the important public health challenges world-wide.[1,2] Dogs act as an essential definitive host while ruminants and other herbivorous animals such as sheep, cattle, goats, and camels as well as human act as intermediate hosts harboring a cyst forming stage, hydatid cyst.[3-5] Humans get infected following accidentally ingestion of *E. granulosus* eggs from infected dog excretions contaminating vegetables, drinking water or through handling infected dogs or their feces.[6] Iraq is considered to be one of the most important endemic country of this disease.[7-11]

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Recently, various strains of the genus *E. granulosus* have been recorded. *E. granulosuss.l.* is includes distinct genotypes that in turn comprising *E. Granulosus* sensustric to (genotype G1–G3 that infect sheep and buffalos),[4] *E. equines* (G4 in horses), *E. ortleppi* (G5 in cattle), and *E. canadensis* (G6/G7, G8, and G10).[12] However the most common strain that have been isolated from human being was G1.[13]

Available evidences have revealed that pathogenesis of the disease has been associated with parasite genotype so genetic characterization of *E. granulosuss. l.* genotype is essential for drug responses and prevention and control of the disease various geographical districts.[5] Based on sequence analysis and further techniques conducted in Iran and other countries, the dominant genotypes include G1-G3 and G6/G7.[14]

So far, treatment of cystic echinococcosis depends upon surgical removal of the cyst and/or chemotherapy (For inoperable cases), depending on different factors including size and location of the developing cysts, viability status (whether fertile or sterile), the interaction between the invading parasite growth and the surrounding host tissue, potential complications following cyst rupture and emergence of secondary hydatid cysts.[15]

Benzimidazole derivatives such as albendazole and mebendazole are widely used anthelminthics, as an alternative to invasive surgery in patients with uncomplicated, disseminated and/ or in operable cases.[16,17] Moreover, those Benzimidazoles are used to prevent the emergence of secondary hydatid cysts following surgical resection or the percutaneous therapy.[18,19] Benzimidazole derivatives destroy the parasite by the inhibition of tubulin polymerization, blockage of glucose absorption, and damaging of the organelles in the germinal layer.[16,20] However, in clinical settings, a proportion of the patients do not respond adequately to benzimidazole treatment.[15,21] Furthermore, subsequent adverse effects including gastrointestinal symptoms, leukopenia, liver toxicity, and allergic manifestations, often occur in long-term administration of the drug.[21,22]

To the best of our knowledge no studies concerning cystic echinococcosis response to albendazole treatment in respect to parasite mitochondrion partial gene polymorphism, has been conducted so far. The current study aimed to investigate the role of mitochondrion partial gene extracted from different isolates of *E. granulosus,* in responsiveness to albendazole treatment in mice experimentally infected with hydatid cyst, that could be used as a biomarker to assess albendazole treatment efficacy.

Methods

Source of Protoscoleces

Hydatid cysts were obtained from infected livers of sheep slaughtered in Erbil Governmental Abattoir and processed as described in our previous study[8] with some modifications. Briefly, hydatid cysts were aseptically dissected and cyst fluid (containing protoscoleces and parasite germinal layer fragments) was aspirated and dispensed in a clean sterile screw cupped centrifuge tube. Protoscoleces were allowed to stand for 20 minutes at room temperature and the fluid was carefully removed. The protoscoleces, obtained as described, were washed Three times with 0.15 M phosphate buffered saline (pH 7.2) containing penicillin (100IU/ ml) and streptomycin (100IU/ml), and assessed for the viability by motility of flame cells and eosin (0.1 %) exclusion test as described by.[23] Protoscoleces from each isolate were separated into two batches. First preserved in a same volume of ethanol (70%) at -20^{o}C for molecular analysis. The second part was used for direct *in vivo* experiment.

Molecular analysis

Genomic DNA extraction

Molecular analysis was conducted in the Department of Biology, College of

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Education, University of Salahaddin. The frozen hydatid cyst tissue samples were removed from the freezer and allowed to thaw at room temperature of 25°C for 30 minutes prior to be subjected into DNA extraction protocol. To remove the ethanol, a volume of 100µL of ethanol fixed protoscoleces was taken from each sample and rinsed three times with sterile distilled water in a 10 ml capacity sterile test tube. The genomic DNA was extracted from the prtoscoleces and germinal layer fragments using the commercially available kit from GeneAll® ExgeneTM for Clinic SV mini kit (South Korea). The DNA extraction protocol was followed according to the leaflet provided with the kit. The concentration and purity of extracted genomic DNA was assessed by Nano-Drop spectrophotometer. The samples that yield a purity of 1.8 to 2.0 were considered for further analysis.

Polymerase Chain Reaction (PCR) and Sequence analysis

A region of 400 bp of the mitochondrion partial genome was amplified using the following primers:

F- 5' AGGGGAGCGTAAGGTTTTGG 3' and R- 5' AAGCACATCGAACCGACCTT 3' (Accession number on NCBI, MG672293.1).

The amplification protocol was carried out according to a previously reported study.[24] Briefly, a final volume of 50 µL containing approximately 50 ng of genomic DNA in the presence of appropriate buffer with 200 µM of each dNTPs, 2.5 mM MgCl2, 50 pmol of each primer and 1.5 units of Taq DNA polymerase (AMPLIQON, Denmark). PCR reactions were performed in all samples with positive and negative controls. The PCR reactions involved an initial 3 min at 95ºC denaturation step, followed by 35 cycles of 60 sec at 95ºC, 60 sec at 56ºC and 90 sec at 72ºC, with a final incubation at 72ºC for 3 min in a PCR ALPHA MAX Thermal Cycler **(**Alpha, United Kingdom). The PCR products were analyzed with 1% agarose gel electrophoresis by Ethidium bromide (BioBasic Canada INC, Canada) which enables high quantum yield and excellent stability of DNA. The results were visualized by UV illumination at (240-366 nm) wavelength on a UV transilluminator, and the gel was documented with a Polaroid photo documentation camera. Amplified DNA bands were purified from the gel using QIAquick Gel Extraction Kit (Qiagen, Germany), following the manufacturer's instructions. The purified amplicons were sequenced in both directions using forward and reverse primers by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Humanizing genomics Macrogen Inc., Seoul, South Korea.

***In vivo* experiments**

Albendazole doses preparation

The stock albendazole solution used for *in vivo* studies was prepared by dissolving 50mg of the pure powder of the drug in 25ml of 0.1% Dimethyl-sulfoxide (DMSO), mixed thoroughly, and then filtrated through a 0.2 µm Millipore filter at room temperature, and preserved at 3-5 °C until used. working albendazole solution was prepared immediately prior to administration by diluting stock solution in distilled water, reaching a final administered concentration of albendazole in a volume of 250µL of distilled water.[25]

Experimental infection and albendazole treatment in murine model

A total of 168 male and female Albino (BALB)/c mice weighing 22-25 gm, were separated into two groups of 6 animals for each parasite isolate (14 isolates). All mice groups were experimentally infected by intraperitoneal injection of 200 µL of PBS pH 7.2 containing 2000 viable protoscoleces. Four weeks post infection one of the two mice groups for each isolate were treated orally (250µL) with 15 mg/kg/ day for 30 days. Second group for each isolate was left as positive control group. Eight weeks after the last dose of treatment, all mice were anesthetized with 12.5mg/kg xylazine (Interchemie, Netherland) and 87.5 mg/kg ketamine (Cluj-Napoca, Romania), and dissected.

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Internal organs were checked for developed hydatid cysts.[7,25]

Ethical consecrations

For Mice breeding, handling and management, World Medical Association statement onanimal use in biomedical research (https://www.wma.net/policies-post/wma-statement-on-animal-use-in-biomedical-research/) was followed. The protocols were approved by the research ethics committee in the College of Medicine, Hawler Medical University (Meeting code: 3, paper code: 2 on 14th February 2024).

Statistical analysis

SPSS (version 25.0) was used to analyze the data. Student's t-test was used for the comparison of the number of developed cysts between the positive, and albendazole treated groups. A one-way analysis of variance test as well as correlation coefficient (r) were calculated among albendazole treatment efficacy (%) in respect to *E.granulosus* mitochondrion partial genome polymorphism. Albendazole treatment efficacy rates were calculated as described by Kim and Sharpless, 2011; Rafiei et al., 2009.[26,27] $P \leq 0.05$ was considered to be statistically significant.

Sequence information was obtained from the NCBI database to characterize *E. granulosus* isolates. The results were analyzed and compared with related *E. Granulosus* sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST). Chromatograms of the amplified region of mitochondrion partial genome were checked using Finch TV software, while MEGA X software was used to edit the amplicon sequences, and to characterize the amplicon sequences corresponding polypeptide chain.

Results

Out of 45 hydatid cysts that were collected from infected sheep at Erbil Governmental Abattoir, 17 of the cyst samples that containing protoscoleces with viability >90 % were subjected into molecular analysis. Following DNA extraction, the 17 DNA samples were amplified by PCR targeting 400 bp of *E.granulosus* mitochondrion partial genome, and sequenced (Figures 1 and 2). The sequencing profile of the isolates revealed no polymorphisms in the isolates 3, 5, 9,12,13, and 14. Minor sequence polymorphisms, 0.33 % were observed in each of the isolates 6, 7, 8, that resulted in corresponding amino acid replacement. Furthermore, remarkable DNA sequence polymorphisms, 2.44%, 2.35%, 2.18%, 1.87%, and 1.35% in the isolates 11, 4, 10, 2, and 1, respectively, causing affectable amino acid replacement in the suggested polypeptide sequences of those studied genes, were observed (Table 1, Figure 3). Three of the 17 isolates revealed sequence profile that did not match corresponding previously published genes on National Center for Biotechnology Information (NCBI) gene bank, therefore those three isolates were excluded from the further molecular analysis as well as from *in vivo* albendazole efficacy testing experiments.

The results of the *in vivo* experiments revealed that mice groups that infected experimentally with the studied isolates, responded variously to the albendazole therapy. Secondary hydatid cysts were significantly reduced in albendazole administered mice infected with isolates1, 2, 4, 5, 6, 7, 8, 13, and 14, comparing with their corresponding positive control groups. Whereas, there was no significant impact of albendazole treatment in mice infected with isolates 3, 9, 10, 11, and 12.

The results was also revealed that the treatment efficacy of albendazole is positively correlated (Correlation coefficient = 0.128) with the polymorphism in *E. granulosus* mitochondrion partial genome (Table 1). Highest albendazole treatment efficacy rate (%) was observed in the isolates 1, 2, 6, 7, and 8 which exhibited DNA sequences polymorphisms of 1.35%, 1.87%, 0.33%, 0.33%, and 0.33%, respectively, comparing with source isolate DNA, clone MG672293.1 (https://www.ncbi.nlm.nih.gov/nuccore/MG672293.1).

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Table 1 Correlation of albendazole efficacy and mitochondrion partial genome polymorphism in experimentally infected albino (BALB)/c mice

Mice Group	Parasite Isolate	Albendazole Efficacy (%) *#	*P* value**	Identity to the Source (MG672293.1) (%) #	Polymorphism (%) *	No. of Replaced Amino acids
1	1	95.83	0.003	98.65	1.35	6
2	2	96.4	0.008	98.13	1.87	14
3	3	41.75	0.056	100	0	0
4	4	85.81	0.013	97.65	2.35	32
5	5	72.08	0.036	100	0	0
6	6	91.30	0.013	99.67	0.33	1
7	7	93.75	0.012	99.67	0.33	2
8	8	96.52	0.012	99.67	0.33	1
9	9	49.75	0.189	100	0	0
10	10	64.99	0.158	97.82	2.18	8
11	11	48.50	0.060	97.56	2.44	21
12	12	63.54	0.055	100	0	0
13	13	75	0.032	100	0	0
14	14	66.67	0.022	100	0	0

* Correlation coefficient = 0.128(association of albendazole efficacy and mitochondrion partial genome polymorphism).
** Student t test (control positive group versus treated group).
#One-way analysis of variance test (*P* <0.001).

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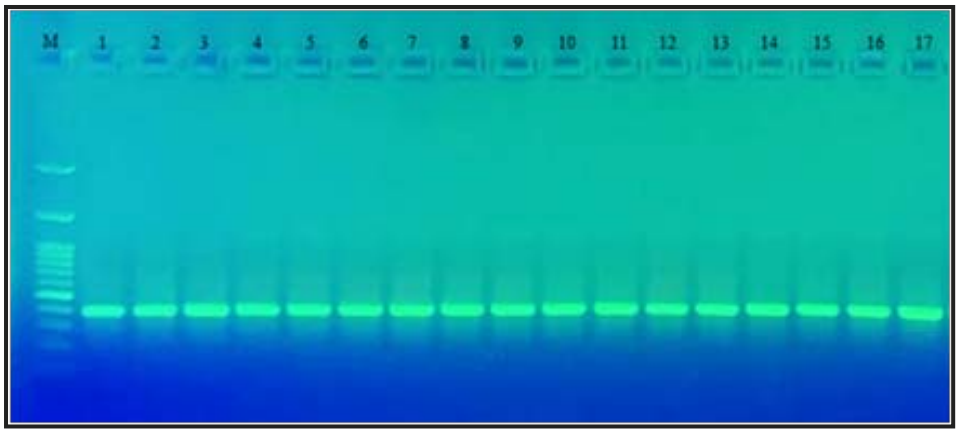

Figure 1 PCR-amplified *E.granulosus* mitochondrion partial genome fragments (400 bp) from 17isolates (lanes 1-17). M: 100 bp DNA molecular weight ladder

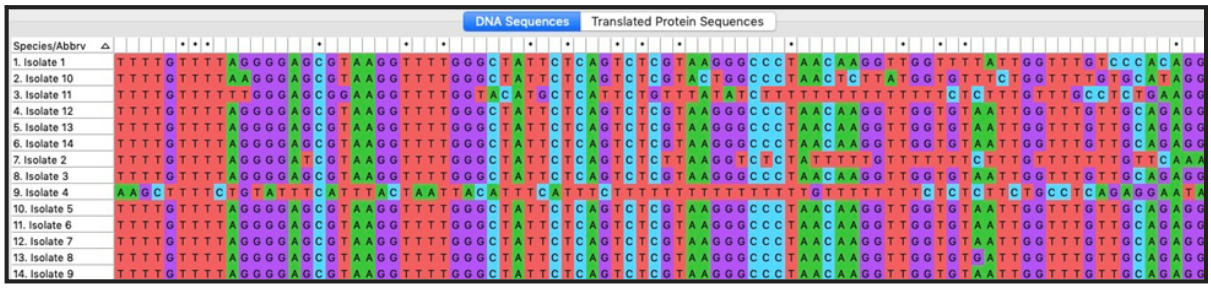

Figure 2 Alignment of DNA sequences of mitochondrion partial genome from 14 isolates of *E.granulosus* hydatid cysts collected from infested sheep livers in Erbil city

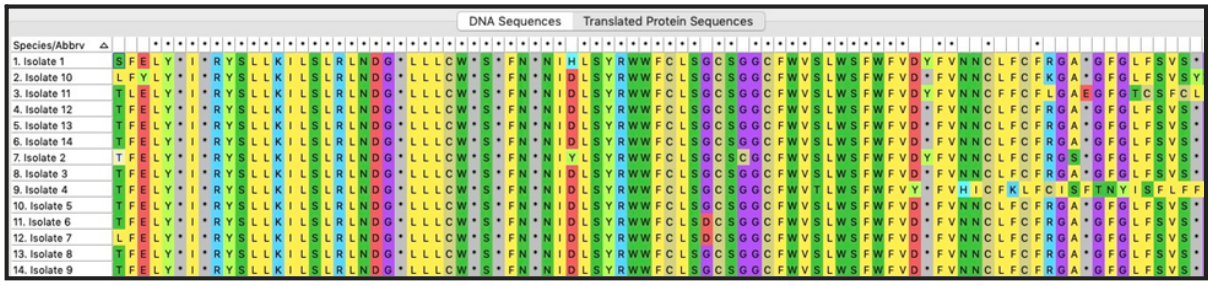

Figure 3 Alignment of suggested polypeptide sequences encoded by mitochondrion partial genome from 14 isolates of *E.granulosus* hydatid cysts collected from infested sheep livers in Erbil city

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Discussion

Biomarkers that could be used to assess the curing efficacy of chemotherapeutic agents is one of the pivotal targets in the clinical setting, since most of the anti-microbial agents trigger undesired adverse effects, as well as prolonged duration of drug administration, which may last up to 6 months, may predisposes to reversable or irreversible toxicity in the host, and may lead to the emergence of resistant strains of the causative pathogen.[28]

Albendazole, a benzimidazole compound, has been shown effective anti-hydatid cyst activity in the intermediate host. However, a proportion of the cases respond very poorly to albendazole therapy.[27] This concept may support the finding that have been obtain from the present study, since out of 14 groups of mice that have been infected experimentally with protoscoleces extracted from 14 separate isolates of sheep liver hydatid cysts, responded variously to albendazole therapy. In the present study the albendazole treatment efficacy rate ranged between 41.75 % and 96.4 % in the 14 groups of mice infested with hydatid cysts from 14 different isolates. The figure was generally higher than that observed by,[27] who recorded efficacy rate only 22.2 % considering the number of the developed cyst in the treatment efficacy rate measuring. This inconsistent might be due to the followed drug administration protocol, the used doses and/ or due to different parasite strains that might be used in both studies.[27,29]

Recently, molecular studies have shown remarkable intraspecific genetic variations in the strains of *E. granulosus* which impact on the various characteristics of the parasite, including transmission, host specificity, biochemical analysis, pathogenicity, immune responses and sensitivity to antihelminth drugs.[29-33]

In the present study sequencing of the PCR- amplified product (400 bp) of the *E.granulosus* mitochondrion partial genome, and that previously characterized by (Kinkar et al., 2018)[33] as *E.granulosus* sensustric to genotype G1 (Accession number, MG672293.1 on NCBI), have shown various polymorphic sequence profiles. Six isolates of those 14 showed a 100% matching with the source (MG672293.1), but they revealed the lowest albendazole treatment efficacy rate which ranged between 41.75 % - 72.08 %, compared to the isolates that revealed polymorphisms in the mitochondrion partial genome that also resulted in the replacement of the corresponding amino acid sequences (Table 1). This finding might lead to the suggestion that the local isolates of *E.granulosus* exhibit higher sensitivity to albendazole, comparing with the source isolate (MG672293.1). Mitochondrion genes were also investigated to be used as a biomarker in the diagnosis of cystic echinococcosis. Moradi et al.[2] investigated the presence of *E. granulosus*-specific DNA in the serum of infected individuals, by detecting two mitochondrion genes, cytochrome c oxidase subunit I and NADH dehydrogenase subunit I.

The finding of that study revealed that *E. granulosus* releases little quantities of the parasite cell-free DNA into the circulatory system. Such finding could be utilized to detect the parasite mitochondrion partial genome polymorphism for assessing the sensitivity of the causative strain of the parasite to albendazole treatment prior to start the treatment regime which may last for up to six months.[22,25]

The present study revealed that 14 isolates that have been collected from sheep livers were clustered within the *E. granulosus* common sheep strain(G1), with sequences identity ranging from 97.56 % and 100% comparing with the reference sequence (MG672293.1) on NCBI. This finding is consistent with the results reported by other authors,[34] who observed that, in Aqrah city, the majority of the amplified products and sequencing profile of a mitochondrial gene, NADH dehydrogenase

subunit I in the isolates that collected from sheep and humans were 95-96% identical to the common sheep strain (G1) (KU169241). Also, they reported four substitution mutations without insertion or deletion. This miracle may be related to the complex and prolonged evolutionary history of *E. granulosus*.[35]

Conclusion

A polymorphic sequence profiles of *E.granulosus* mitochondrion partial genome can be used to detect the sensitivity of the causative strains to albendazole. However, further studies must be conducted to involve hydatid cyst isolates from other intermediate hosts, and to assess the effectiveness of other anthelmintic drugs against various strains of the tapeworm *E.granulosus*.

Competing interests

The author declares that he has no competing interests.

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