Nad1 gene analysis of *Echinococcus granulosus* from sheep in Aqrah city, Iraq

R.N. Hamoo[1], N.G. Mustafa[2*] and S.A. Abdulraheem[3]

[1] Department of Biology, College of Education for Girls, [2] Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Mosul, Mosul, [3]Veterinary hospital in Aqrah, Dohuk, Iraq
*nashaat_ghalib@yahoo.co

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Abstract

Echinococcus granulosus (*E. granulosus*) is a dog tapeworm cestoda; it is larval stage responsible to cystic echinococcosis, one of the most common and dangerous worldwide zoonotic parasitic disease. The aim of this study was the molecular identification of the local strain of *E. granulosus* isolated from sheep liver slaughtered in the principal abattoir of Aqrah city, Northern of Iraq during Jun-Nov. 2017. In this study, 37 sheep liver infected by *E. granulosus*, 12 of high DNA purity fertile (have protoscolices) cyst of them were considered. A molecular study conducted on the mitochondrial NADH dehydrogenase 1 (nad1) gene. Results demonstrated that *E. granulosus* isolates were sheep strain (G1) genotype, with fascinating highly corresponding 95% and 96% to global isolates, particularly to north African and Mediterranean countries, by employing phylogenetic tree analysis. So, the isolates of our project were deposited in Genbank (accession No. MG792129). This study findings provide that the local isolates of *E. granulosus* from sheep liver in Aqrah city, Northern of Iraq are loyally equivalent to global strains and isolates, in addition, nad1 gene considers a perfect biomarker in a molecular identification and phylogenetic study of this parasite.

Keywords: Nad1 gene, *Echinococcus granulosus*, Hydatid cyst, Phylogenetic tree, Sheep
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تحليل جين nad1 للمشوكة الحبيبية من الأغنام في مدينة عقرة، العراق

رضاء ناظم حمو[١]، نشأت غالب مصطفى[٢] ، سمير أحمد عبد الرحيم[٣]

[١] قسم علوم الحياة، كلية التربية للبنات، [٢] فرع الفسلجة والكيمياء الحياتية والأدوية، كلية الطب البيطري، جامعة الموصل، الموصل، [٣] المستشفى البيطري في عقرة، دهوك، العراق

الخلاصة

ديدان المشوكة الحبيبية هي من الديدان الشريطية التي تصيب الكلاب، ويسبب الطور اليرقي لهذا الطفيلي داء الأكياس المائية، وهو من أكثر الأمراض المشتركة خطورة بين الإنسان والحيوان. هدفت الدراسة الحالية إلى التمييز الجزيئي للعزلة المحلية للطور اليرقي لطفيلي المشوكة الحبيبية، والمعزولة من الأكياس المائية من كبد الأغنام المذبوحة في المجزرة الرئيسية لمدينة عقرة في شمال العراق، خلال الفترة من شهر حزيران - تشرين الثاني لسنة ٢٠١٧. تم الحصول على ٣٧ كبد مصاب بالأكياس المائية، اختير منها ١٢ عينة مخصبة (لاحتوائها على الرؤيسات) ولنقاوة DNA العالية فيها، وأجريت الدراسة الجزيئية على جين أنزيم المايتوكوندريا NADH dehydrogenase 1 (nad1). أظهرت النتائج أن العزلة المحلية هي النمط الجيني لعزلة الأغنام (G1)، ومن خلال استعمال نتائج الشجرة التطورية ظهر تقارب كبير ٩٥% و ٩٦% مع العزلات العالمية وخاصة لمناطق شمال أفريقيا والشرق الأوسط. كما تم في هذا البحث تسجيل العزلة المحلية في بنك الجينات العالمي (رمز التسجيل MG792129). نستنتج من هذه الدراسة أن العزلة المحلية للمشوكة الحبيبية المعزولة من كبد الأغنام في مدينة عقرة في شمال العراق لها تشابه كبير مع العزلات العالمية فضلاً عن أن الجين nad1 يعد مؤشر حيوي ممتاز في الدراسة الجزيئية والتطورية لهذا الطفيلي.

Introduction

Cystic echinococcosis (hydatidosis) is a well-known cosmopolitan zoonotic illness caused by the larval stage (metacestode) of a dog tapeworm *Echinococcus granulosus* (*E. granulosus*) (1), and transmitted from a definitive host (usually dogs and other canids) to the intermediate hosts (including human, sheep, cattle, camel, and other mammals) through contaminated foods or water (2). *E. granulosus* cyst is a spherical fluid-filled cyst that involves the internal germinal layer of cells reinforced by a typical non-cellular laminated membrane of different thickness (3). A sheet of granulomatous adventitia, host-produced, borders the cyst. While brood capsules are small vesicles that bud internally from the germinal layer and create several protoscolices by an asexual multiplication (4).

A growing concern to echinococcosis perhaps due to it is global distribution, troublesome diagnosis and control, expensive and complicated treatment strategy (may require surgical intervention to remove cysts), and in progressive cases, it could be fatal (5). According to the epidemiological studies, traditional ten genotypes G1-10 of *E. granulosus* were recorded (6). The findings of molecular genetic studies of *E. granulosus* mainly obtained by using mitochondrial DNA (mtDNA) (7,8). The molecular analysis is according to the complex of host species/parasite genotype, followed by numerous taxonomical kinds like; *Echinococcus granulosus sensu lato* (*E. granulosus s.l.*) that cause cystic echinococcosis, and *E. granulosus sensu stricto* (*E. granulosus* s.s.). Ten strains of *E. granulosus* were identified depending on the genetic characterization G1-G10, this includes sheep strain G1, Tasmanian sheep strain G2, buffalo strain G3 (9), *E. equinus* G4, *E. ortleppi* G5 cattle strain, *E. canadenisis* G6-G10, G6 cattle strain, G7 pig strain, and *E. felidis* (lion strain) that is supposed to occur only in Africa.

In our country Iraq, a large number of studies attended to *E. granulosus*, but only a few of them explored molecular characteristics. An interesting study by Saeed *et al*. (10) explores the prevalence of hydatid cyst in the north of Iraq during 1990-1998, they recorded a high ratio of *E. granulosus* infection in sheep 15%, in cattle 10.9, and goat 6.2. Remarkably, they found that fertile cysts percentage in sheep was 64%, while in goat it was 35.7, and in cattle 29.8. The authors also documented half of stray dogs 49.5% were have an adult worm of *E. granulosus* (10). Although cystic echinococcus is endemic in the north of Iraq (10,11), but only a little attention has been paid to the molecular study of the nad1 gene of this parasite in Iraq. Therefore, this article aims to molecular identification of the local strain of *E. granulosus* isolated from sheep liver in Aqrah city, Northern of Iraq.

Material and methods

Sample collection

Thirty-seven samples of sheep (both sex) liver were collected during June-November 2017, through a routine inspection process of slaughtered animals in the central abattoir of Aqrah city, Kurdistan region of Iraq. Sheep were from the Aqra city center (36°44'29"N 43°53'36"E) and near rural areas. According to Cao *et al*. (3) and under sterile conditions, protoscolices and cyst fluid were aspirated via sterile G21 syringe, one sample from each cyst was considered of high purity DNA, and fertile (have protoscolices) after light microscope inspection. Samples were washed three times by sterile phosphate buffer saline (PBS), and well-kept in 70% ethanol (v/v) at 4°C temperature until DNA extraction.

DNA extraction

Mitochondrial DNA (mtDNA) was extracted from hydatid fluid included protoscolices, using commercial DNA extraction kit (PrimePrep™, Genet Bio, Korea), manufacturer instructions were followed, and the purity of the extracted DNA was screened by nanodrop (Thermo-Fisher Scientific, USA). Extracted DNA was preserved at -20 °C temperature to PCR amplification.

PCR and electrophoresis

In the present project, 488 bp fragment of mitochondrial NADH dehydrogenase 1 (nad1) gene was amplified using specific primers (F: 5'-CGTAGGTATGTTGGTTTGTTTGGT-3', R: 5'-CCATAATCAAATGGCGTACGAT-3') (Integrated DNA technologies /USA). In the free ddH_2O the primers dissolved to achieve the final concentration of 100 pmol/µl as a stock solution to prepare 10 pmol/µl as work primer suspended, 10 µl of the stock solution was added to 90 µl of the ddH_2O to attain a final volume of 100 µl.

PreMix PCR kit (i-Taq, 2525 Intron, Korea) composed of 5 µl Taq PCR PreMix [reaction buffer (10 X), i-Taq DNA Polymerase (5 U/µl), dNTPs (2.5 mM), gel loading buffer (1 X)], F primer 10 picomols/µl and R primer 10 picomols/µl, volume extracted DNA 1.5 µl (as a negative control, 1.5 µl of ddH_2O instead of DNA samples were used), 16.5 µl of ddH_2O, completing of the final volume of PCR reaction was 25µl. The mixture was amplified by thermal cycler machine (MultiGene™ OptiMax Gradient, Labnet, USA) under the conditions that shown in table 1 (8).

Separation of the PCR products was done on a 2% agarose gel electrophoresis (CBS, Scientific, USA), at 5 volt/cm^2. TBE buffer 1x (IBS.BT004, Conda, USA) for 90 min., and exposure to 302 nm ultra-violate light, illuminator (Vilber lourmat, France) to visualized after ethidium bromide staining (Intron, Korea).

Table 1: PCR conditions of 488 bp fragment of the mitochondrial nad1 gene of *E. granulosus*

Phase	Temperature (°C)	Time	No. of cycle
Initial denaturation	95^{0}C	5 min	1
Denaturation	94^{0}C	30 sec	
Annealing	56^{0}C	30 sec	35
Extension	72^{0}C	50 sec	
Final extension	72^{0}C	10 min	1

Sequence analysis

PCR product (15 µl) and forward and reverse primers were used to the sequencing of 12 samples of *E. granulosus* isolated from sheep liver. The sequencing process was done depending on nicem (the national instrumentation centre for environmental management) online (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab apparatus is DNA sequencer (3730XL, Applied Biosystem). BioEdit programs and BLAST (Basic Local Alignment Search Tool) were employed to the homology exploration, that available at ncbi (National Centre for Biotechnology Information) website at (http:// www.ncbi.nlm.nih.gov) (12).

Results

In the present study, 12 samples of *E. granulosus* were isolated from 37 naturally infected sheep livers, slaughtered in the central abattoir of Aqrah city, Kurdistan region, Iraq, during June-November 2017. DNA was extracted from fertile cysts, own protoscolices, and purity of DNA was confirmed by A_{260}/A_{280} ratio of 1.8-2.0. By using specific primers (listed in materials and methods, successful PCR amplification of 488bp fragment of mitochondrial NADH dehydrogenase 1 (nad1) gene of *E. granulosus* was accomplished. PCR amplified fragments were established by electrophoresis, while there were no bands on the gel in the negative control (Figure 1). Analysis of alignment via online blast demonstrates correspondence of 95 % (8 samples) and 96 % (4 samples) of an examined sequence fragment when compared with the database of Genbank of *E. granulosus* accessible data (Table 2). Also, it was evident and interesting that there are four codons substitution mutations; nucleotides: 5-7 (AAT to TCT), 14-16 (TAC to TGT), 20-21 (GTT to GGT), and 26-28 (TTA to GGT) of an investigated fragment (Table 2 and Figure 2). One of the fascinating novel outcomes of this study, the sequence of nad1 of *E. granulosus* was G1 genotype and verified as accession No. MG792129, Ver. MG792129.1 in GenBank(https://www.ncbi.nlm.nih.gov/nuccore/MG792129).

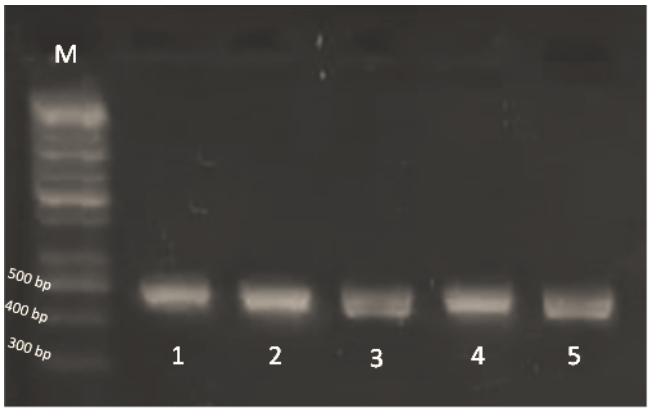

Figure 1: Agarose gel electrophoresis of PCR amplicon of NAD1 gene, using 2% agarose, 80 V, 70 Am for 2 hrs., (lanes 1-5: 488 bp *E. granulosus* isolates; M: 100 bp DNA ladder).

Table 2: Genetic and SNP analysis of nad1 gene of *E. granulosus* from sheep liver in Iraq

	Accession	Isolates and strains	Country	Source *E. granulosus*	Compatibility	Expect	Score	Range
1	ID: KU169241.1	-	Tunisia	nad1 gene	96%	8e-117	416	434 to 691
2	ID: KJ162554.1	G15	Iran	nad1 gene	96%	8e-117	416	342 to 599
3	ID: JX217907.1	SC-Y3	China	nad1 gene	96%	8e-117	416	348 to 605
4	ID: EF367323.1	NG3	Morocco	nad1 gene	96%	8e-117	416	434 to 691
5	ID: KX039965.1	MEX1	Mexico	nad1 gene	95%	1e-115	412	4781 to 5038
6	ID: KX039964.1	CHI4	Chile	nad1 gene	95%	1e-115	412	4781 to 5038
7	ID: KX039960.1	BRA6	Brazil	nad1 gene	95%	1e-115	412	4781 to 5038
8	ID: KX039953.1	ARG17	Argentina	nad1 gene	95%	1e-115	412	4781 to 5038
9	ID: KU925433.1	ALB2	Albania	nad1 gene	95%	1e-115	412	4781 to 5038
10	ID: KU925431.1	ROM1	Romania	nad1 gene	95%	1e-115	412	4781 to 5038
11	ID: KU925430.1	GRE1	Greece	nad1 gene	95%	1e-115	412	4781 to 5038
12	ID: KU925429.1	FIN1	Finland	nad1 gene	95%	1e-115	412	4781 to 5038

Score	Expect	Identities	Gaps	Strand
416 bits(460)	2e-112	247/258(96%)	0/258(0%)	Plus/Plus

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Query  1    GTATAATTTGTTGTACACCGTTTGATTATGGTACAACAATTATTCATTTTTAAGGTCGGT  60
            ||||  |||||||| ||  || |||| |  ||||||||||||||||||||||||||||||
Subject434  GTATTCTTTGTTGTGTACTGGTTGAGGTGGTTACAACAATTATTCATTTTTAAGGTCGGT 493

Query  61   TCGATGTGCTTTTGGATCTGTTAGGTTTGAGGCTTGTTTTATGTGTGTGGTGATTTTTTG 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject494  TCGATGTGCTTTTGGATCTGTTAGGTTTGAGGCTTGTTTTATGTGTGTGGTGATTTTTTG 553

Query  121  TGCTTTGTGTAGTTGTAGGtataatttaattgatttttattataattgttgattaagttt 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject554  TGCTTTGTGTAGTTGTAGGTATAATTTAATTGATTTTTATTATAATTGTTGATTAAGTTT 613

Query  181  gttattatttccattaatttatgtgttatttttaatatgtATATTGTGTGAAACTAATCG 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject614  GTTATTATTTCCATTAATTTATGTGTTATTTTTAATATGTATATTGTGTGAAACTAATCG 673

Query  241  TACGCCATTTGATTATGG                                          258
            ||||||||||||||||||
Subject674  TACGCCATTTGATTATGG                                          691
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Figure 2: Alignment statistics for match with reported G1 reference sequence of *E. granulosus* isolate Tataouine nad1 gene, sequence ID: KU169241.1 Length: 824.

The phylogenetic tree was designed according to our Iraqi isolate of *E. granulosus* compared with best identical global strains/isolates. Results make evident that the most likeness to this study isolate was Tunisian, Iranian, and Moroccan (Figure 3).

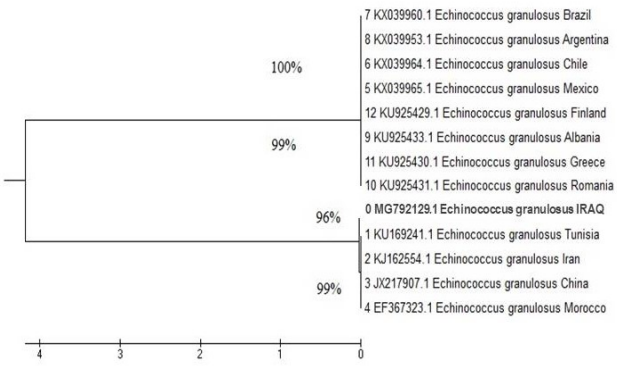

Figure 3: Phylogenetic tree of *E. granulosus* with international isolates established on nad1 sequence from *E. granulosus* of sheep liver in Iraq.

Discussion

Cystic echinococcosis is still the most significant and critical worldwide zoonotic disease, and it is documented in all areas of North Africa and the Middle East (12). Recently, there is global necessity and curiosity to molecular study and identification of various parasitic diseases, accordingly, mitochondrial genes of *E. granulosus*, particularly nad1 and cox1, was considered a well-established vital molecular biomarker to categorize the genetic diversions of *E. granulosus* isolated from man, sheep, cattle, and various species of animals (13,3).

In human and animals, the most predominant genotype in the areas of Middle East countries, including Iraq has been described as G1 genotype (14-18). Our results agree with the previous results as Fadhil and A'aiz (19), in which they recorded that the genotypes of isolated samples from sheep are G1 80%, and G3 20%. While Hammad *et al.* (20), investigated for the first time other than G1 genotypes in Iraq in Kirkuk and Sulaimania cities. On the other hand, Baraak *et al.* (21), and Muhaidi *et al.* (22) demonstrated that G1 (sheep strain) genotype was the predominant isolate of various organs of human and cow, respectively. However, authors believed that G1 could produce unusual lesions like that of E. *multilocularis* (23). The predominance of G1 genotype (sheep strain), in this article results agree with most previous studies (24), and this may indicate that G1 strain is prevalent in endemic areas like Iraq in both man and animals. However, the high number of sheep may play a role in this manner. On the other hand, the diversity of nucleic acid was significant and there were four substitution mutations without insertion or deletion. This miracle maybe related to the complex and long evolutionary history of *E. granulosus* (25,26).

The evolutionary history of *E. granulosus* investigated by phylogenetic tree analysis via Tamura-Nei model (27), accordingly, the degree of relation with global isolates can be predicted. Our findings are promising and should be explored with other future works focus on the molecular

identification and phylogenetic analysis of *E. granulosus* from various hosts in many areas of Iraq. The *E. granulosus* isolated from the liver of sheep in Aqrah city, Northern of Iraq is always corresponding to the global strains and isolates, and nad1 gene could be considere a great biomarker in the molecular identification and phylogenetic study of this parasite.

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