MOLECULAR CHARACTERIZATION OF *ECHINOCOCCUS GRANULOSUS* ISOLATED FROM HUMAN HYDATID CYST USING MITOCHONDRIAL *COX1* GENE SEQUENCING IN DOHUK PROVINCE- KURDISTAN REGION, IRAQ

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ABSTRACT

To characterize the circulating *E. granulosus* genotype in Duhok province, a total of 17 human (14 female and 3 male) hydatid cysts (10 from lung and 7 from liver) were subjected to molecular analysis using mitochondrial *cox1* gene sequencing. Total DNA was extracted using Qiagene DNeasy blood and tissue mini kit then it was electrophoresed on 1.5% Agarose gel in TBE 0.5% buffer for 20 minutes and sequenced. Variable sequences of 418bp were obtained for 15 out of 17 samples. The sequences were aligned with old sequences of G1, G2, G3 genotypes and the new Turkish and Iranian genotypes. As a result, the Duhok isolates were more identical to the newer Turkish and Iranian G1 common sheep genotype sequences than the old ones due to the occurrence of different multiple nucleotide substitutions among nearly half of Duhok isolates. In conclusion, the results of this study showed the presence of the G1-G3 cluster sensu stricto cluster genotype in Duhok province, therefore preventive measures should be arranged accordingly.

KEYWORD: *E. granulosus, Strain identification, G1 Sheep strain, Microvariants Genotype.*

INTRODUCTION

Hydatid disease (CE) is caused by the larval stage of the genus *Echinococcus*. This disease is considered as one of the most important helminthic infection creating significant public health and economic problems with a worldwide spread (Budke *et al.,* 2006; Thompson, 2008). CE affects humans and has a worldwide prevalence of six million cases (Siracusano *et al.,* 2009). In Iraq, reports ranging from prevalence rate of 10-96 cases/year among human (Molan, 1993; Saeed *et al.*, 2000; Duhok Azadi Teaching hospital statistics, 2009 and Abdullah, 2010)) to infection rate among animals of 12.3-15-6%, 1.4-6.2%, 1.6%-10-9% among sheep, goats and cattle respectively (Saeed *et al.,* 2000; Al-Nakeeb 2004; Ghaffar, 2008; Abdullah, 2010). On the other hand, much higher prevalence rates have been reported among dogs which ranged from 38- 79.1% (Molan and Saida 1989; Molan and Baban, 1993).

To date, 10 genotypes (G1-10) within *E. granulosus* have been identified and this categorization follows very closely the pattern of strain variation emerging based on biological characteristics (McManus, 2004: Nakao *et al.,* 2007 and 2010; Thompson, 2008 and Moro and Schantz, 2009). The level of genetic differences between recognized species of Echinococcus are not appreciably greater than those between strains (Bowles *et al*., 1992; Bowles and McManus 1993a and Bowles *et al*.,1995). All the genotypes of *E. granulosus*

except the dog/horse (G4) and the Finnish cervid (G10) strains have been found to infect humans (OIE, 2008). Due to the presence of more than one livestock animals in our locality as well as the presence of more than one strain in neighboring countries, such as in Iran G1, G3 and G6 (Zhang *et al.*, 1998; Sharbatkhori *et al.*, 2010 and Pour *et al.*, 2010), in Turkey G1 and G3 (Vural *et al.*, 2008 and Ergin *et al.*, 2010), in Jordan G1 and possibly G4 (Al Qaoudy *et al.*, 2003). Therefore, it is necessary to conduct this study in order to identify the *E. granulosus* strain(s) circulating in this Province and in the light of obtained results the controlling program will be suggested.

MATERIALS AND METHODS

Sampling

A total of 17 human lung and liver hydatid cysts were collected during the period from January- December 2010 from the surgical theaters of 4 hospitals covering the 6 Duhok districts (Zakho, Summel, Amedi, Shekhan, Akre and Bardarash). These districts have 958000 population and they cover an area of 6553km^2. The source and locations of HCs are shown in Table (1).

Table (1). Showing the number, the host gender and the site of the isolated hydatid cysts from Human

S.N	Host gender	Liver site	Lung site	Total number
1	Human (male)	0	3	3
2	Human (female)	7	7	14
	Total	7	10	17

The surgically resected intact and or ruptured hydatid cysts (Figure.1) were kept in normal saline and transported to the central laboratory. In the laboratory the tissue of each cyst was washed with PBS and divided into 2 parts, one part was preserved in 10% formalin for histopathological studies, the other part containing the germinal layer with protoscolecies / or without protoscolecies was stored in sterile capped containers containing about one volume (v/v) of 70% ethanol at -40°C to be used later for DNA extraction.

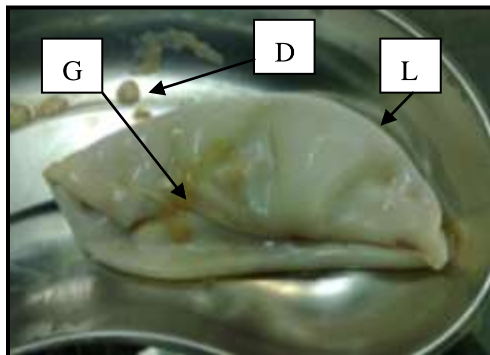

Figure (1) Opened echinococcal lung lesion resected from a 9 year old boy (Stained with E&H) (L: laminated membrane. G: Germinal layer, D: Daughter cyst)

Microscopic Examination

For Histopathological study, the tissue were processed, permanent slides were prepared and stained with Eosin and Hematoxylen according to John and Alan (1991) techniques to observe histological characteristics of *Echinococcus* (the typical nucleated germinal layer, the acellular laminated layer and the presence / or absence of protoscolices).

Molecular study

1. DNA extraction

The germinal layer and or protoscolices were washed 3 times with PBS pH 7.2 to remove the ethanol; 25 mg of the protoscolices and or germinal layer were used for total DNA extraction using DNeasy blood and tissue extraction kit (Qiagen/ Germany) according to manufacturer instructions. The DNA concentration and purity were measured by Nanoodrop 2000-UK.

2. PCR process

For genotyping the partial mitochondrial

Cytochrome oxidase subunit 1 (pmt *cox*I) gene fragment was amplified using AB Applied Biosystem Thermal Cycler 2700 version 2.0. The amplification was carried out at the Central laboratory of Duhok, also at the Department of infectious parasitic and immunomediated diseases, Rome, Italy and at Sichuan CDC research laboratories/ China.

Specific primer sets were designed in Invitrogen Company Shanghai China. A forward primer sequence (5´TTT TTT GGC CAT CCT GAG GTT TAT3´) and a reverse primer (5´TAA CGA CAT AAC ATA ATG AAA ATG3´) to amplify (444 bp) pmt *cox*1 were used as previously described by Bart *et al.* (2006); Casulli *et al*. (2008) and Pour *et al*. (2010). For PCR run, the lyophilized primers from Invitrogen biotechnology are reconstituted in 10 times volume TE buffer according to manufacturer instructions to get 100µMs stock concentration, Go *Taq* green master mix Hot start polymerase enzyme ready for use is provided from Invitrogen biotechnology, 25µls PCR reaction mixture is prepared by mixing of 12.5µl *go Tag* green mater mix, 1µl 10mMs each of the forward and reverse primers, 1µl DNA template and 9.5µls deionized water.

The thermal cycler condition was arranged as described previously(Bowles *et al*., 1994) with slight modifications with initial denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds with final extension cycle at 72°C for 7 minutes. For PCR product visualization 3µl of PCR aliquot is electrophoresed in agarose gel 1.5% in 0.5% TBE buffer using 2µl DNA ladder 100bp for comparison and stained with gold view for visualization. Electrophoresis carried out using Cleaver scientific M-P 300N electrophoresis machine on 100v for 20 minutes, then visualized using UV illuminator (Bio Rad). The obtained bands are shown in Figure (2).

A 20µls PCR aliquot with 80µls forward and reverse primers sequence were sent to GenLab ENEA Casaccia, Rome, Italy and Invitrogen-Shanghai laboratories, China for sequencing.

RESULTS AND DISCUSSION

The prepared slides from 17 human HCs previously diagnosed by ultra sound and or X-ray were examined under microscope using 100X oil immersion power. The lesions were characterized by the presence of a typical thick pinkish laminated layer and a highly nucleated inner germinal layer, protoscolices and brood capsules were observed in fertile cysts which include 4/ 10 of the cysts isolated from lungs and 4 /7 liver cysts. The rest (9) liver and lung cysts were sterile Figure (2 A and B), showing sections of fertile (A) and sterile (B) HCs.

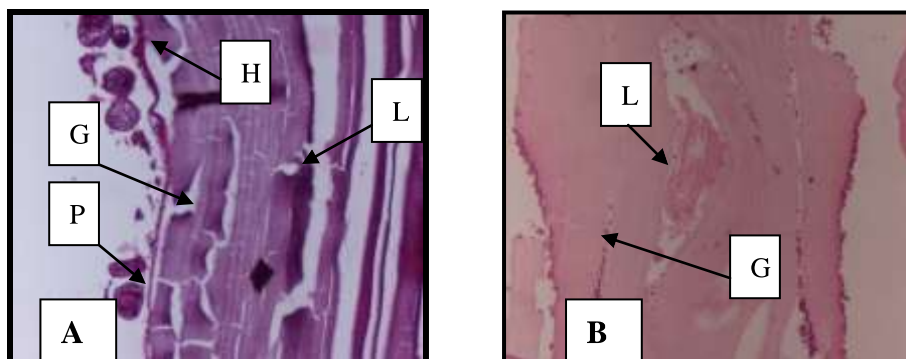

Figure (2. A and B) Microscopical appearance of *E. granulosus* lesions. (H&E stained, magnification 100X) A: Fertile HC; B: Sterile HC, P: Protoscolices, G: Germinal layer, L:

laminated layer, H: Hocks.

All samples were identified morphologically as *E. granulosus* cysts. The sterile cysts characterized by the absence of protoscolecies and brood capsules. The sterility may be due to unsuitable growth media (Eckert and Deplazes, 2004), or different strains of *E. granulosus* might cause variation in the fertility rate in various environmental regions (McManus, 2006).

Molecular Studies

DNA was extracted successfully from all the 17 samples. The lowest concentration obtained was 1.6 ng/ µl, whereas, the highest concentration was 68.8 ng/ µl, the lowest and highest levels of purity were 0.1 and 2.4 at A_{260}/A_{280}, respectively (Table. 2). All samples gave clear DNA bands except sample (S9) which gave a hazy band Figure (3).

Table (2). The concentrations and purity of extracted DNA of the 17 HC samples

Sample No.	DNA concen. ng/µl	Purity A260/A280
1	11.7	1.82
2	68.8	1.85
3	13.2	2.12
4	5.4	1.9
5	6.8	1.5
6	25.3	1.8
7	7.1	1.3
8	3.5	0.1
9	40.5	1.84
10	6.1	1.7
11	8.5	1.4
12	14.8	1.72
13	2.8	1.9
14	83.2	1.79
15	17.1	1.7
16	2.6	1.45
17	7.2	1.56

Figure (3). A PCR product electrophoresis pattern of 17 human HCs samples electrophoresed on 1.5% Agarose gel.
(NC: Negative control, PC: Positive control, S1- S17 are HC samples)

The obtained PCR products were directly sequenced, the forward and reverse sequences were assembled and 418bp fragments were obtained for 15 samples which were later used in analysis. Alignment was done for obtained samples that gave 7 different groups; which were S1, S2, S3, S4, S5, S7, and S17. Multiple alignments for 15 samples were done with reference sequences of G1, G2, G3, G4 or G5 genotypes previously described by Bowles *et al.*, (1992). Later, they were aligned again with newer Turkish and Iranian reference of Vural *et al.,* (2008) and Pour *et al.,* (2010) using DNASTAR Laser-gene software version 11.
During alignment, they showed percent identity level of 98.9-99.7% with G1 common sheep strain (accession number M84661) ; 99.2-99.5% with G2 Tasmanian sheep strains of Bowles et al., (1992) (accession number M84662) and 98.6-99.5% with G3 (accession number M4663). Afterward, during alignment with recent Iranian and Turkish G1 sequences they showed higher percent identity of 99.0-99.7%.
Therefore, all of the 15 isolates considered to be G1common sheep strain microvariants as shown in Table (3)

Table (3) Characteristics and Percent identity of Duhok HC isolates based on pmt *cox1* alignment with Australian G1of Bowles *et al*. (1992), Iranian G1of Pour *et al*. (2010) and Turkish G1[3] Cluster, Vural *et al* . (2008) Reference sequences

(accession number M84662) and	Host name	Location	Fertility	Percent Identity			
				Bowles *et al*., (1992) G1	Iranian Pour *et al*., (2010) G1	Turkish G1[3] Cluster	Strain
S1	Human	Lung	-	98.9	93.0	99.3	G1
S2	Human	Lung	+	99.5	99.8	99.8	G1
S3	Human	Lung	-	99.2	99.5	99.5	G1
S4	Human	Lung	+	99.2	99.5	99.5	G1
S5	Human	Lung	+	99.7	99.5	99.0	G1
S6	Human	Lung	-	99.5	99.8	99.8	G1
S7	Human	Liver	+	99.7	99.5	99.0	G1
S8	Human	Liver	+	99.5	99.8	99.8	G1
S9	Human	Lung	-	Sequence failure			
S10	Human	Lung	-	99.5	99.8	99.8	G1
S11	Human	Liver	+	99.2	99.5	99.5	G1
S12	Human	Lung	-	99.5	99.8	99.8	G1
S13	Human	Liver	-	Sequence failure			
S14	Human	Lung	-	99.5	99.8	99.8	G1
S15	Human	Liver	+	99.2	99.5	99.5	G1
S16	Human	Liver	-	99.5	99.8	99.8	G1
S17	Human	liver	-	99.2	99.5	99.5	G1

During the alignment with reference G1 genotype of Bowles *et al.* (1992), 7 out of 15 sequences showed 1- 4 nucleotide substitutions, such as either C → T; T → C; G → A or A→ G at 7 positions (38 , 62, 78, 88, 185,250 and 279). The sample number (5) showed 3 nucleotide substitutions, S1 and S7 showed 2 nucleotide substitutions, the other 4 samples showed only 1 substitution. The other 8 sequenced samples did not show any nucleotide substitutions (Table 4). This result confirmed the occurrence of different mutations in Iraqi isolates, which is similar to that reported in Turkey by Vural *et al.* (2008) and in Iran by Sharbatkhori *et al.* (2010).

Table (4). The types and sites of nucleotide substitutions Among Duhok HC isolates

Sample Number	Nucleotide substitution	Nucleotide position	Nature of cyst
1	C → T	78	Sterile
	G → A	185	
4	A → G	250	Fertile
5	A → G	62	Fertile
	C → T	88	
	T → C	279	
7	A → G	38	Fertile
	T → C	279	
11	C → T	78	Fertile
15	C → T	78	Fertile
17	C → T	78	Sterile

The results of the present study confirm the presence of G1common sheep strain and according to the new taxonomy of the *E. granulosus* (Nakao *et al.,* 2007; Thompson, 2008; Vural *et al,* 2008 and Pour *et al.,* 2010), the 15 Duhok isolates were considered to be G1-G3 cluster sensu stricto cluster genotype.

In conclusion, this study confirmed the occurrence of G1-G3 sensu stricto cluster genotypes in Duhok province, since these genotypes have a wide range of livestock animal intermediate hosts; therefore the epidemiological measure should be arranged accordingly.

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پوخته

ژ بو سالوخەتدانا نفشێن *E. granulosus* ل پارێزگەها دهوکێ، کومەکا ژ 17 کیسکێن ئاڤێ ژ مروڤان (14 ژن و 3 زەلام) کو (10 ژ پشا سینگی و 7 ژ مێلاکێ) هاتنە شلوڤەکرنا مولیکیولی و خواندنا زنجیرا جینی یا جینێ *cox1* یێ مایتوکوندرایێ . هەمی ترشێ ناوکی هاتە ئینانەدەر بکارئینانا کیتێ Qiagene DNeasy blood and tissue mini kit و هاتە electrophoresis کرن لسەر 1.5٪ جیلێ ئەگاروز دناڤ 0.5٪ TBE تفتیدا بو دەمێ 20 دەقیقا. زنجیرێن هەمەجور پێکهاتی ژ 418 بنکێن جوت هاتنە دەستڤەئینان بو 15 نموونا ژ کوما 17. و زنجیرە هاتنە بەراورد کرن دگەل زنجیرێن کەڤن یێن نفشێن کەڤن ژ جورێ G1, G2, G3 و نفشێن نوی یێن تورکی و ئیرانی. دئەنجامدا نموونێن دهوکێ نێزیکتر بون بو نفشێن نوی ژ جورێ نفشێ پەزی ێ بەربەلاڤ ژ نفشێن کەڤن ئەوژی ژ ئەگەرا رویدانا گهورینێن هەمەجور یێن جینی دناڤ نێزیکی نیڤا نموونێن دهوکێدا. ئەنجامێ ئەڤێ حواندنێ دیارکر هەبونا نفشێ G1-G3 cluster sensu stricto cluster genotypeکو جورێ تەنێیە ل پاریزگەها دهوکێو لەورا دڤێت پینگاڤین خوپاراستنی بهێنە رێکخستن لدویف ئەنجامان.

الخلاصة

تناولت هذه الدراسه تحديدالانماط الجينيه لاكياس دوده المشوكات الحبيبيه *E. granulosus* المعزوله من 17 مصابا (14 انثى و3 ذكور) في محافظة دهوك. حلل الدنا المحيي لجين بيوت الطاقه ال *cox1* من هذه الاكياس باستخدام طقم تحليل الدم والانسجه من شركة كياجين الالمانيه. عرض للترحيل الكهربائي electrophoresis على هلام الاكاروز بتركيز 1.5% في داريء فوسفات ال TBE لمدة 20 دقيقه، تم الحصول على سلاسل من القواعد النتروجينيه بطول 418 قاعده مزدوجه ل 15 عينه. قورنت هذه السلاسل بالسلاسل المعروفه للانماط G1, G2, G3 وكذلك مع الانماط الحديثه لكل من تركيا وايران وتبين ان عزلات دهوك كانت اقرب للانماط الجينيه لسلالة الاغنام الموجوده في كل من تركيا وايران والتي تختلف عن النمط القديم للانماط القديمه وذلك بحدوث استبدال في تسلسل القواعد النتروجينيه عند بعض المواقع التي تم تحديدها في هذه الدراسه في حوالي نصف من هذه العزلات وتعد هذه الدراسه كاول دراسه في العراق . وباختصار اظهرت هذه الدراسه وجود النمط الجيني G1-G3 cluster sensu stricto cluster genotypeفي محافظة دهوك لذلك يجب تصميم برامج السيطره للحد من انتشاره.