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Gene Sequencing of Hydatid Cysts Isolated from Human and Sheep in Central Euphrates Provinces, Iraq

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التسلسل الجيني للأكياس العدارية المعزولة من الإنسان والأغنام في محافظات الفرات الأوسط ، العراق

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ABSTRACT

Background:

Hydatid cystic disease is one of the most prevalent zoonosis diseases between people and animals. It develops in several organs, the most significant of which is the liver and lung as a hydatid cyst in numerous hosts, including humans. It causes many complications that may result in death. There are no safe and efficient medicines for this parasite in use, and the research process is ongoing to find such treatments.

Materials and Methods:

During the period from November 2021 to May 2022, collected 18 samples of hydatid cysts (9 human samples and 9 sheep samples) were examined. DNA isolated from a germinal layer, and amplified of the product using the technique of PCR and sequenced in gene 18S rRNA Internal Transcribed Spacer (ITS1) and (ITS2), which has a molecular weight of (1,100 and 750) base pairs. The studied samples were matched with the samples installed in the gene bank.

Results:

The results found that there were samples that matched the isolate of the gene bank with different percentages. Human Hydatid Cysts which chosen to draw the evolutionary tree, where the percentage of matching was 99 %.

Conclusion:

The presence of the parasite was verified by using the genes ITS1 and ITS2 and then drawing the evolutionary tree after matching the studied isolate with the isolate registered in the NCBI and the molecular techniques have significantly improved our comprehension of the variety and distribution of *E. granulosus.*

Key words:

Echinococcus granulosus, Gene sequencing, (ITS1), (ITS2).

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الخلاصة

المقدمة:

يعد مرض الكيس العدريات أحد أكثر الأمراض حيوانية المصدر انتشارًا بين البشر والحيوانات. يتطور في عدة أعضاء، وأهمها الكبد والرئة ككيس عداري في العديد من المضيفين، بما في ذلك البشر. يسبب العديد من المضاعفات التي قد تؤدي إلى الوفاة. لا توجد أدوية آمنة وفعالة لهذا الطفيل قيد الاستخدام، وعملية البحث جارية لإيجاد مثل هذه العلاجات.

طرق العمل:

خلال الفترة من نوفمبر 2021 إلى مايو 2022 ، تم فحص 18 عينة من الأكياس العدارية (9 عينات بشرية و 9 عينات من الأغنام). عزل الحمض النووي من طبقة جرثومية، وتم تضخيمه للمنتج باستخدام تقنية PCR والمتسلسل في الجين S rRNA18 داخلي نسخ فاصل (ITS1) و(ITS2)، الذي له وزن جزيئي (1,100 و 750) أزواج قاعدية. ومن ثم تم مطابقة العزلات المدروسة مع العينات المثبتة في بنك الجينات.

الاستنتاجات:

تم التحقق من وجود الطفيل باستخدام الجينات ITS1 و ITS2 ثم رسم شجرة التطور بعد مطابقة العزلات المدروسة مع العينات المسجلة في NCBI، وقد حسنت التقنيات الجزيئية بشكل كبير فهمنا لتنوع وتوزيع الحبيبات المشوكة.

الكلمات المفتاحية:

المشوكة الحبيبية، التسلسل الجيني، (ITS1)، (ITS2)

INTRODUCTION

Hydatidosis caused by *Echinococcus granulosus* larval stage is a zoonotic disease and one of the most important for human and domestic animals [1, 2 and 3]. This disease leads to many medical, veterinary and economic problems. Iraq is regarded as one of the countries plagued by the endemic sickness of hydatidosis [4, 5 and 6]. Even though that CE is a serious parasite illness that is a growing public health problem, the present there hasn't been much progress in efforts to control the disease globally, therefore enhancing clinical management, medications, and diagnostics [7].

Depending on the genetic indicators of the mitochondria and nucleus, there has been a high level of genetic diversity for granular of the *E.granulosus* in recent decades, Different genotypes or strains for *E.granulosus* that identified, these strains are distinguished by a diverse range of hosts, pathogenicity, parasite maturation style, epidemiology, chemotherapeutic sensitivity, and disease treatment and prevention techniques [8].

A little over a decade ago, the gene in question was utilized to identify Trematodes strains and kinds. There have been some attempts to utilize it to diagnose tapeworm strains and species due to its high accuracy [9 and 10].

The genotypes of *E.granulosus* were determined based on and controlled molecular genetic analysis using the basis of DNA sequencing [11,12 and 13]. Therefore, the aim of the study is to molecular identifies the most *Echinococcus granulosus* that common strains in central Euphrates provinces, as well as to study the gene sequences of the strains, draw the phylogenetic tree, and compare them to the strains recorded in NCBI-BLAST for determining the most that common strains for humans and sheep from the central Euphrates provinces.

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MATERIALS AND METHODS

- **Collection samples of hydatid cysts from humans and animals:**

The samples of humans were collected from Al-Qadisiyah and Al- Najaf Provinces and other private laboratories, as well as hospitals in Babylon and Baghdad provinces, the hydatid cyst samples, were taken from infected humans preserved in formalin solution (10%) who had previously been diagnosed by ultrasound (sonar) or CT scan and had undergone surgical procedures. These samples were delivered right away by the specified container to the college of Science for Women's parasitology lab at Babylon University. [14 and 15].

Hydatid cyst samples were taken from diseased sheep carcass organs. at abattoirs in Babylon and Al-Najaf provinces. The samples were packed in a sealed and labeled polyethylene box and sent to the laboratory. The protoscolices were collected and stored in a conservative medium Kreb Ringers solution and Hydatid fluid (4:1) respectively. The viability of the protoscolices was accomplished assessed using the eosin stain 0.1% approach [16].

- **DNA isolation, amplification, and sequencing:**

Total cellular DNA was isolated from samples of hydatid cyst fluid and germinal layers and purified with the help of Geneaid\ Taiwan, according to the manufacturer's instruction. In both human and animal samples, PCR was used to detect genotypes of the *E.granulosus* based on the 18SrRNA-ITS1 gene. This method was used to implement this technique [17]. The method outlined by Gareh et al. [18]. based on (ITS1-BD1 Forward 4S reverse, ITS2−3S- forward A28–reverse) genes were used to carry out this method that amplification by using the PCR technique, provided by South Korea in the following table (1):

Table 1: Name of the primer and its sequence of nitrogenous bases

Primers		Sequence	Amplicon
ITS1-BD1 Forward 4S (reverse)	F	5′GTCGTAACAAGGTTTCCGTA-3′	1,100 bp
	R	5′TCTAGATGCGTTCGAATGTCGATG-3′	
ITS2−3S- forward A28- reverse	F	5′GGTACCGGTGGATCACTCGGCTCG3′	750 bp
	R	5′GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3′	

Each reaction was performed in 25µl solution containing (Maxime PCR PreMix Kit) 12.5µl, the primer forward 10 p/mol 2µl, the primer reverse 10 p/mol DNA 2µl, 50 ng/µl of the DNA template 5µl, deionized distilled water 3.5µl). All reactions were optimized to be performed under the same conditions: hold at 95°C for 5 min, hold for 5 min at 95°C, followed by 40 cycles with the denaturation step for 60 s at 95°C, the annealing step for 60 s at 60°C and the extension step for 180 s at 72°C. On a 1.5 percent agarose gel, the amplified DNA products were resolved and stained with ethidium bromide (10 mg/L). and the photographed with the transmitted UV light.

The products of DNA replication were sent to the South Korean Macrogene company to sequence the nitrogenous bases of the studied sample and the results were compared with the BLAST program and Bio Edit program with the genes registered in the NCBI gene bank, After obtaining the sequence of the genetic bases of the DNA of the products of molecular polymerization, they were analyzed and compared with the data obtained from the NCBI Gene Bank, the studied type was registered within the gene bank, the sequence of the local human sample of *E.granulosus* no. 2 strain was alignment with references strains deposited in a gene bank, the tree was drawn based on NCBI data references online.

RESULTS AND DISCUSSION

The PCR technique's results demonstrate the success of all DNA amplification procedures retrieved from the protoscolices and the germinal layer of the ITS1 and ITS2 genes in detecting the presence of the parasite. A result has appeared ITS1 positive in some samples of hydatid cysts of sheep numbered (1, 3, 4, 5, and 8) at the molecular weight (1100) bp shown in Figure (1), and negative results in human (2, 6 and 7) after doing electrophoresis on an agarose gel, ITS1 appeared in sheep samples but did not appear in human samples. The reason may be due to a defect during the DNA extraction method, or if the amount of DNA is small.

Figure (2) Presence of the diagnostic gene for (ITS1 and ITS2) gene, ITS1 gene at the molecular weight (1100bp) and ITS2 gene at the molecular weight (750bp). Sample (1 and 2) of human hydatid cysts, ITS1 had negative results in the sample (1) and positive results for ITS2. But sample (3 and 4) sheep hydatid cysts had positive results for ITS1 for sample (3) and ITS2 for sample (4). This study is consistent with the study [19]. to detect the presence of the parasite using ITS1gene.

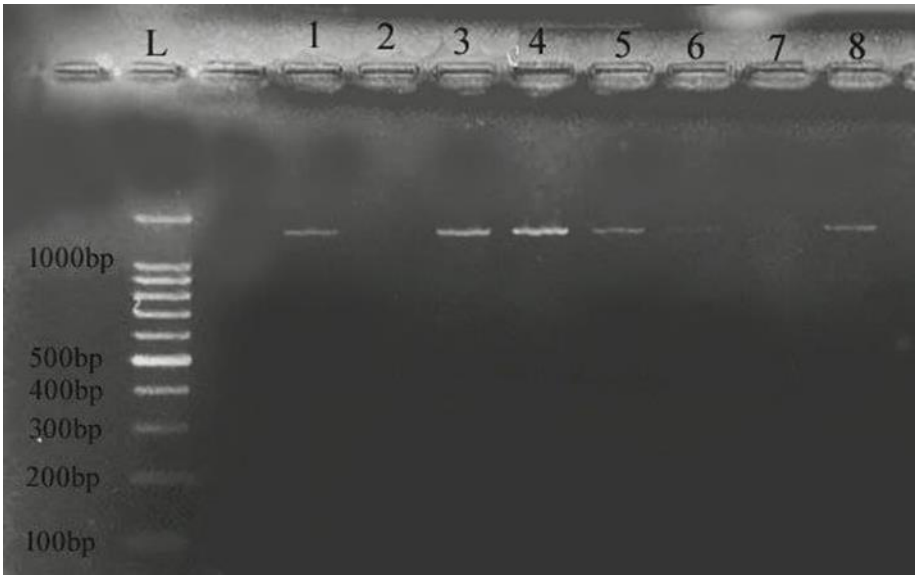

Figure (1): Banding patterns of ITS1 gene in hydatid cysts, L; molecular size marker (100 bp); ITS1 gene in samples of sheep hydatid cysts numbered (1, 3, 4, 5 and 8) at the molecular weight (1100bp), (2, 6 and 7) in human after electrophoresis done on the agarose gel.

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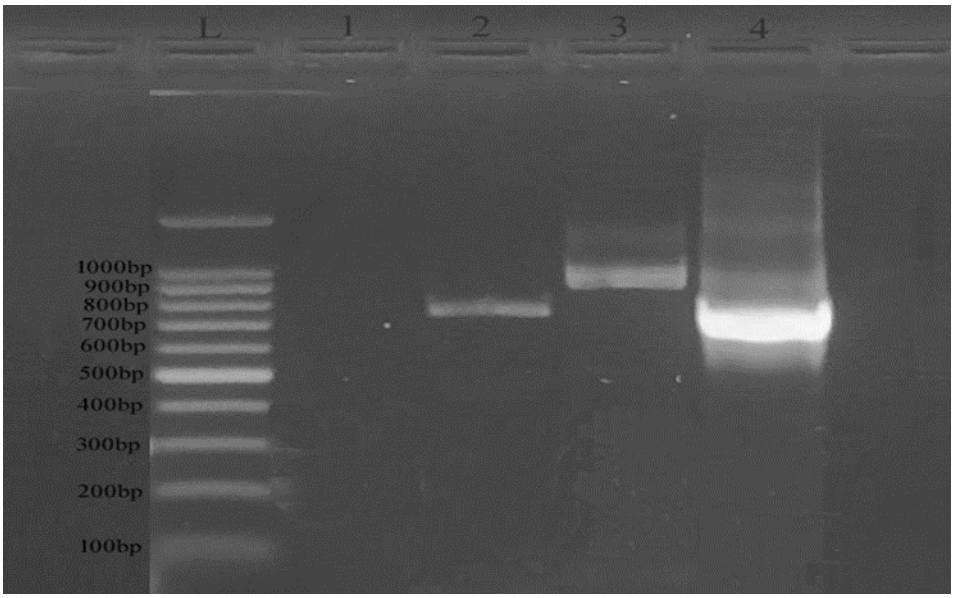

Figure (2): Banding patterns of ITS1 and ITS2 gene in hydatid cysts with molecular weight (1100 and 750) respectively, L; molecular size marker (100bp), Line 1 and 2 in human hydatid cysts, 3 and 4 sheep hydatid cysts.

Figure (3): Shows Phylogenetic tree analysis of local *E.granulosus* isolates utilized for genotyping based on partial sequences of the ITS2 gene. After matching this sample with the samples from the gene bank. The study reached the identification of a sample of hydatid cyst at the molecular level using the region ITS, and the matching percentage was 99% with a sample which has ID: KX434757.1 named *E.granulosus* from India and this is recognized through the alignment drawn to compare it (Figure 4).

The Tamura-Nei model tree approach and the maximum likelihood method were used to create the phylogenetic tree in (the MEGA X version). The *E.granulosus* isolate from human No. 2 shown in figure (2) showed genetic variation related to NCBI-BLAST *E.granulosus* isolates at genetic changes compared to the other samples under investigation that were represented in the phylogenetic tree, Additionally, samples that registered with the NCBI Blast in a diameter of 0.1 had a genetic variation that was nearly identical to the recorded international sample.

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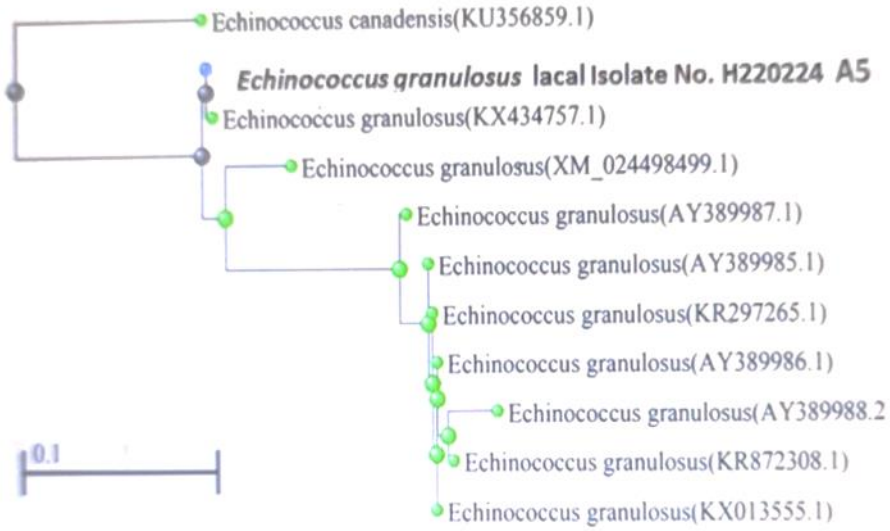

Figure (3): A phylogenetic tree for the isolate samples used in the current investigation was created and compared to another foreign isolate listed in NCBI.

Echinococcus granulosus isolate VT-ASg 5.8S ribosomal RNA gene and internal transcribed spacer 2, partial sequence

Sequence ID: KX434757.1 Length: 619 Number of Matches: 1

Range 1: 1 to 619 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1133 bits(613)	0.0	617/619(99%)	0/619(0%)	Plus/Plus

```
Query  28   ATTAATGTGAATCGCAGACTGCTTTGAACGTCGACATCTTGAACGCATATTGCGGCCATG  87
Sbjct  1    ............................................................  60

Query  88   GGCTTGCCTGTGGCCACGTCTGTCCGAGCGTCGGCTTATGAACCATCACTGTGTGCAATG  147
Sbjct  61   ............................................................  120

Query  148  AGCGGTGGCTGGGGAGAGTGCGGTGCCGTCCCGTGCCGTGGCGCGCGGTGGGTGGAGCGT  207
Sbjct  121  ............................................................  180

Query  208  GTGCTGGTTGGCTGGCTCACGGTGCGGACTGGCGTGGCTTCTCACTAGGTGTGCTGGTGC  267
Sbjct  181  ............................................................  240

Query  268  TGTCGAATTCGGTGGCGTGGAGTTTGCGGTTGTGCTGCTGCAGTGGCCGCAGTGCGGTCA  327
Sbjct  241  ............................................................  300

Query  328  GCCGTTGCGCTGTGCTGTGGCGTTGATGCGCGTGTACGGCAGTTGTGCCAGTAGGTTGGC  387
Sbjct  301  ............................................................  360

Query  388  GGGTGGTGATGCGGTTGCAGTCTTCGCAGTCCACGGACCGTGGCCCAGTGTGCGAATGGG  447
Sbjct  361  ............................................................  420

Query  448  CAGgtgtatgtgtgtgtaggtgtgAGCACGCTGCACACGTTATGTGGATTGTGGATGC  507
Sbjct  421  ............................................................  480

Query  508  TGTGGCGGGTGGGGCGTGCTTTCTTCTCTCGCTCGCCGCAAGCACTTGCATCGTTGTGCT  567
Sbjct  481  .....................................G......................  540

Query  568  GCGTTGCACGTGTAATGCGATGGGTGGGAGGGTGCGCGTCACGCCCCGCCTCGCCTAGAC  627
Sbjct  541  ............................................................C.  600

Query  628  TTGCCTTGATTGACTCGTT  646
Sbjct  601  ...................  619
```

Figure (4): Alignment of the DNA nitrogenous base sequences of *E.granulosus* from India has ID: KX434757.1

The current study constantly undergoing genetic alteration through the appearance of mutations in the region ITS2. Genetic variation is present, and genetic evolution is clear within the species under study H220224 no.2 the similarity ratio of the sample was 99% with the Indian sample ID: KX434757.1 With a little difference between the two variants, only one A\G at position 521 is transition and A\C at position 559 is transition mutation occurred in it, as shown in figure (4). the samples approved in drawing the evolutionary tree showed something indisputable that there is an internal development that leads to variation, so it becomes clear, that *E.granulosus* (KU356859.1) differs greatly from the rest of the samples deposited in the gene bank , *E.granulosus* (XM-024498499.1) is different from the rest of the isolates and our isolate diagnosed in *E.granulosus* H220224 no.2, and even the samples that appear in one group, *E.granulosus* (XM-024498499.1, AY389987.1, AY389985.1, KR297265.1, AY389986.1, AY389988.2, KR872308.1, KX013555.1) appear to be one group, but there is a genetic variation inside it, so they are not on one line.

The different sample sizes may be what's causing the results to differ. their number, origin and timing of collecting, varying geographic regions, types of animal feeding there, or the presence of dogs close to the slaughterhouses and butcher shops that sell or slaughter a particular type of these animals (cattle, sheep), contaminating water and crops with their feces, in addition to the habits and practices of the locals and the degree of health consciousness they possess, and the amount to which intermediate and final hosts have diffused among them, or it can be as a result of some strains in particular locations adapting more than others [20]. emphasized that the goal of studying genetic evolution was to identify the forms of *E.granulosus* that have spread throughout the country, which is crucial information in the fight against this animal illness

The nature of the environment, temperature and climatic conditions all play a role in the emergence of such variations in the samples. Therefore, the current study showed that sequence analysis is simple, fast and very reliable and can be used as an effective tool for identifying and classifying the studied species. It also provided a lot of useful genetic information about the studied species for the management of genetic assets and high efficiency [21].

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Conflict of interests.

There are non-conflicts of interest.

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