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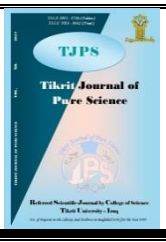

Multiplex PCR Technique for Simultaneous Detection and genotyping of *E. granulosus*, *E. multilocularis*, and other *Taeniidae* in some intermediate hosts in Erbil Province

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ABSTRACT

One of the most significant helminthozoonosis in Iraq and around the world is cystic echinococcosis, which occur by the etiologic agent of *Echinococcus granulosus* and it's distinguished by considerable intra-specific variability (genotypes G1–G10). DNA was extracted from 48 isolated cysts (25 sheep, 12 goats and 11 human) and used as templates to amplify using Trachsel method to differentiate Taeniide (*Echinococcus spp.* and/ or *Taenia spp.*) and *mitochondrial cytochrome c oxidase subunit one (COX1)* gene for genotyping. The PCR products were sequenced, and sequence analysis was used to further evaluate the data. Out of 48 separated of the host cysts, 36 isolates displayed the G1 genotype (100%), and in 12 samples, a nucleotide substitution at position 58 (T→C) results in polymorphism (99.9%). The G1 is considered that the most contagious and prevalent genotype of *E. granulosus* in the world. Our investigation revealed that, a single genotype that may be in charge of the disease's infectivity in sheep, goats, and human as well as its persistence in endemic areas. these epidemiological findings could be guided the successful hydatidosis control measures in Erbil Provence

استخدام تقنية تفاعل البلمرة المتسلسل المتعددة للكشف عن *E. granulosus* و *E. multilocularis* و *Taeniidae* الأخرى في الأغنام والماعز والبشر في محافظة أربيل

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الملخص

أحد أهم أمراض الديدان الطفيلية في العراق وحول العالم هو داء المشوكات الكيسي، والذي يحدث بواسطة العامل المسبب لمرض المشوكة الحبيبية ويتميز بتباين كبير داخل النوع (الأنماط الجينية G1 – G10). تم استخراج الحمض النووي من 48 كيسًا معزولًا (25 خروفًا و12 ماعزًا و11 إنسانًا) واستخدم كقوالب للتضخيم باستخدام طريقة Trachsel للتمييز بين Taeniide (Echinococcus spp. و/أو Taenia spp.) والوحدة

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الفرعية الأولى من السيتوكروم c أوكسيديز الميتوكوندريا (1COX). الجين للتنميط الجيني. تم تحليل تسلسل ناتج تفاعل البلمرة المتسلسل، وتم استخدام تحليل التسلسل لمواصلة تقييم البيانات. من بين 48 عزلة من الكيسات المضيفة المنفصلة، اظهرت 36 عزلة النمط الجيني 1G (100٪)، وفي 12 عينة، أدى استبدال النوكليوتيدات في الموضع 58 (T → C) إلى تعدد الأشكال (99.9٪). يعتبر 1G النمط الجيني الأكثر وجودا" وانتشارًا نوع E. granulosus في العالم. كشفت دراستنا أن نمطاً جينياً واحداً قد يكون مسؤولاً عن عدوى المرض في الأغنام والماعز والإنسان وكذلك استمراره في المناطق الموبوءة. يمكن أن تكون هذه النتائج الوبائية بمثابة دليل على التدابير الناجحة لمكافحة الداء المائي في محافظة أربيل.

1- Introduction

Carnivorous mammals, mostly dogs but occasionally cats, are infected by the tapeworm *Echinococcus spp.*, which serves as the intermediate host. Cystic mass lesions are most usually caused by the extraintestinal larval stages of *Echinococcus species* in the liver, although they can also affect almost all other organs. Humans serve as an accidental and typically "dead-end" intermediate host for echinococcal infections, unless they are eventually consumed by a canine predator to complete the life cycle [1]. As a result of decreasing animal productivity, this disease poses a severe threat to remnants health in numerous rural places worldwide [2], and the infection is obtained through oral uptake of eggs from the final host's feces. The oncosphere larvae emerge from the eggs, breach the intestinal wall, and were circulated in many organs where they settle and growth into the metacestode stage. Eventually, the metacestode produces a significant amount of protoscolices [3]. Understanding the patterns of worm transmission in various geographic areas by CE genotyping is crucial for developing effective control measures and assessing the differences in pathogenicity [4,5]. According to genetic classification, which is mostly based on the homology of the sequences of the two mitochondrial genes cytochrome C oxidase subunit 1 (COX1) and reduced nicotinamide adenine dinucleotide subunit 1 (NAD1), ten different strains have previously been genetically characterized [6]. Five different species have been identified as existing within the E. granulosus species complex by modern taxonomic classification. The genotypes of these include E. granulosus s.s. (G1-G3), E. equinus (G4), E. ortleppi (G5), E. canadensis (G6-G10), and E. felidis [7, 8]. The G1 genotype, often known as the "sheep strain," can infect humans, goats, cattle, camels, and pigs in addition to causing viable cysts in sheep [9]. In order to evaluate the genetic diversity of Echinococcus granulosus sensu stricto (s.s.) metacestodes from four European countries, the DNA sequence analysis of the COX1 gene was used [10]. The objective of the current study was to identify the species of Echinococcus present in the intermediate hosts in Erbil Provence by using multiplex PCR to allow the comparative analysis of three nucleotides and also to sequence the PCR-amplified mitochondrial COX1 gene of hydatid cysts isolated from sheep, goats, and humans in order to establish their genotype.

2- Materials and methods

Animals and human cystic echinococcus (CE) were collected during the period from July 2022 to June 2023.

2.1- Samples Collection:

The entrails of all slaughtered sheep and goats were examined precisely examined for the existence of CE, when a cyst had been seen; all information about the slaughtered livestock and infected organs were recorded on Questionnaire form. A total of 37 animals (25 sheep and 12 goats), including 10 pulmonary and 27 hepatic cystic echinococcus were studied. Furthermore, patients with CE who were admitted to two teaching hospitals (Rizgary Teaching Hospital and Komary Teaching Hospital) provided cyst samples in Erbil Province. The geographic origin and cyst location of patients were noted. Human CE were obtained from 11 patients undergoing surgical operation aging 13–69years, they included: 8 hepatic and 3 pulmonary CEs. The germinal layer of the cysts from human were taken after surgical operation and kept in 70% ethanol and stored at refrigerator (2-8^{o}C) until used.

2.2- Molecular assay

Twenty five mg of germinal layers from animals and human cysts were used for DNA extraction, using the procedure of [10, 11]; from which 80-100µl was obtained, using Bio- Tech Korea, observing the guidelines provided by the manufacturer instructions. PCR product was amplified using Go Taq (Promega, Madison, WI, USA). To define the species of CE; Multiplex PCR method was used with Primer sequence of: $Cest_1$ *E. multiocularis* (5'-TGCTGATTTGTTAAAGTTAGTGATC-3'), $Cest_2$ *E. multiocularis* (5'-CATAAATCAATGGAAACAACAACAAG-3'), $Cest_3$ Taenia spp. (5'-YGAYTCTTTTTAGGGGAAGGTGTG-3'), $Cest_4$ *E. granulosus* (5'-GTTTTTGTGTGTTACATTAATAAGGGTG-3') and $Cest_5$ Taenia spp. (5'-GCGGTGTGTACMTGAGCTAAAC-3') to amplify 123bp for the *E. granulosus*, 275bp for the Taenia spp. and 400bp for *E. mutlilocularis*. The amplification reaction mixture (30µl) consisted of 13.6µl of (2X) Go-Tag master mix, 14.4µl of primer mix (12 pmole) (1.2 µl of primers $Cest_1$, $Cest_2$, $Cest_3$, $Cest_4$ and 9.6 µl of primer $Cest_5$) and 2 µl of template DNA. The amplification reaction was performed

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using AB Applied BioSystem thermo-cycler (Veriti 96 Well Thermal Cycler- Singapore), under the following conditions: a pre-amplification step at 94°C for 5minutes, followed by 40 cycles with denaturing at 94°C for 30s, annealing at 58°C for 90s and extension at 72°C for 10s, and for the final elongation of DNA strands, an ending-extension at 72°C for 5 min. Water instead of DNA was included in each set of PCR reactions as the negative control. For genotyping of extracted DNA fragment, *Cox1* gene was amplified by PCR using a genus-specific primer set (5'- TTTTTTGGCCATCCTGAGGTTTAT-3' and 5'-TAACGACATAACATAATG AAAATG -3'). The PCR mixture was prepared in a 50µl volume containing 2µl template DNA, 25µl of (2X) Go-Tag master mix, 25 pmol (2.5 µl) of each primers (foreword and reverse). The amplification was performed by adding 1 cycle at 94°C for 5minutes before the 35 cycles of the PCR (Hot Start PCR) and adding 1 cycle at 72°C for 5min after the 35 cycles. The PCR parameters were as follows: 35 cycles of initial denaturation for 30s at 95°C, annealing for 30s at 55°C and elongation for 30s at 72°C). The PCR products was performed in 1.5% agarose and visualized by UV tans-illumination (Bio-Rad Gel Doc.). The same PCR product was submitted commercially (Macrogen Inc. South Korea) to sequencing by using the same primers *cox1* gene in both directions and The BLAST method (http://www.ncbi.nlm.nil.gov/BLAST/) was used to compare the nucleotide sequence to the NCBI database.

2.3- Nucleotide Sequence and Phylogenetic Tree Analysis

The species of *Echinococcus* was confirmed by analyzing the nucleotide sequences of the *cox1* gene. The sequences were aligned through the ClustalW algorithm within UGENE [12], provided by BioEdit v7.2.5 [13], with sequences available in the GenBank (NCBI) database. Phylogenetic analyses were performed on individual partial gene sequences using MEGA software (Molecular Evolutionary Genetics Analysis 3.1; (http://www.megasoftware) and the neighbor-joining were used to build the tree.

3- Results

The multiplex PCR amplification for species specific was successfully performed on the 48 CE isolated from different intermediate hosts and the acquired data revealed that all single and multiple cysts (germinal layers) isolated from all intermediate host were *E. granulosus* which was 123bp as expressed in figure 1. Regarding the genotyping of extracted DNA using *cox1* gene; all positive samples confirmed as *Echinococcus granulosus* yielding 444pb, corresponding to the mitochondrial *cox-I* gene as shown in figure (2).The DNA sequences generated of 36 (75%) samples were 100% identically to the sequences of *E. granulosus s.s.* (G1) under the accession number (LC476648 and MT318687) in Genbank and 12 (25%) with *E. granulosus* (G1BC) due to nucleotide changes (T→C) at position 58 as shown in figure 3. The ClustalW application within UGENE was also used to perform the multiple alignments of the nucleotide sequences as indicated in figure 4, it was discovered after drawing the phylogenic tree that all of the samples of hydatid cyst belonged to the G1 and G1BC strain.

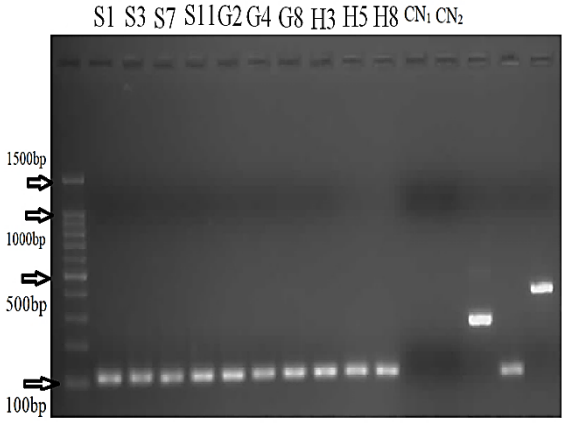

Fig. 1: Multiplex PCR of *Echinococcus* spp. and *Taenia* spp isolates of intermediate host from Erbil Provence. In the multiplex PCR only the 123 bp fragment amplified, indicating that the intermediate host could be infected with *E. granulosus*. DNA marker size (100- bp DNA ladder), Cn_1=Control DNA extraction, Cn_2=Control negative PCR, lanes S1, S3, S7, S11, G2, G4, G8, H3, H5 and H8–specific product for *E. granulosus* isolated from intermediate hosts. Notes: S= Sheep, G= Goats, and H= Human)

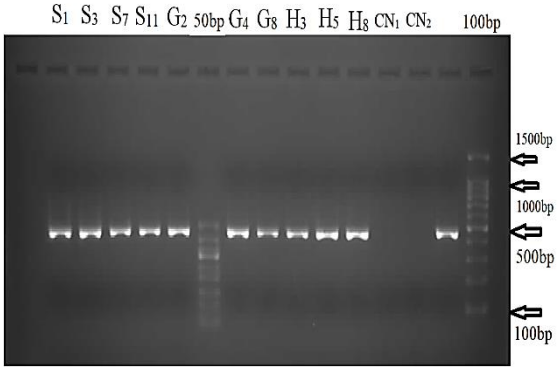

Fig. 2: Gel documentation of PCR results of *E. granulosus* template DNA using mitochondrial cytochrome C oxidase subunit 1 gene.

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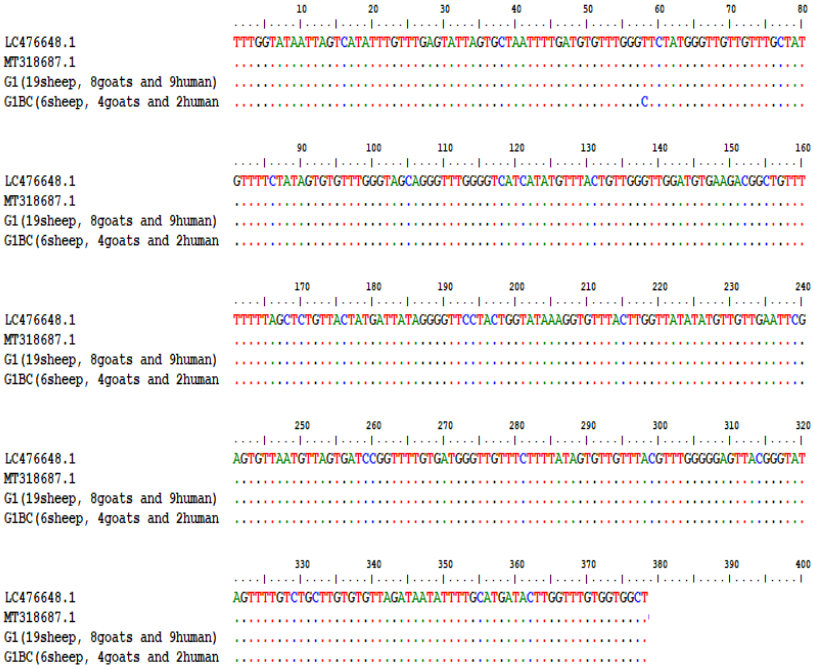

Fig. 3: Genotyping of *E. granulosus* in various hosts identified by mitochondrial analysis in Erbil Province

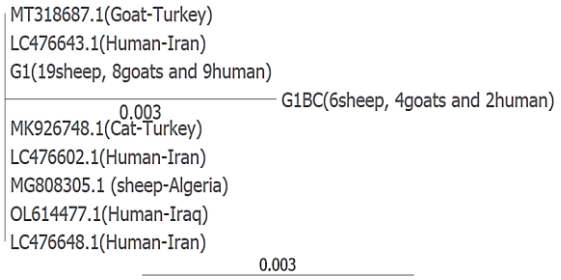

Fig. 4: Maximum likelihood bootstrap tree revealed the relationships of the haplotypes of *cox1* gene fragment for *E. granulosus* isolates with those submitted in Genbank

4- Discussion

Multiplex PCR, a very sensitive technique, was developed by [14] and it was employed to separate *E. granulosus* complex, *E. multilocularis*, and others *Taeniidae* from fecal samples, but in the present study, it has been used with some modifications for the identification of germinal layer of CE and it was successful for this genus. Because multiplex PCR method is unequivocal for the distinguish of taeniidae (*Echinococcus spp.* and *Taenia spp.*) from intermediate host [15], and furthermore, the primers used to identify *E. granulosus complex* and *Taenia species* are not completely specific for the species that they are meant to detect. Additionally, primers can find certain non-taeniid cestodes and the E. granulosus primers also amplify DNA from the recently identified *E. shiquicus*, *E. vogeli*, and *E. oligarthrus* [16].

Several molecular methods have been used to distinguish the numerous *Echinococcus strains/species* [17] . The mitochondrial *cox1* gene was used in this study because it is one of the markers used to distinguish different strains, and *cox1* was helpful in identifying *Echinococcus* variations [18]. The phylogenetic analysis of concatenated sequences showed that the amplified DNA had 100٪ identity with the *COX1* gene of E granulosus (G1-genotype). The results of this investigation were agreed with the results of other investigator like [19] in Iran and [20] in Turkey, reported that the majority of the samples (19 sheep, 8 goats and 9 human) were 100% identity with human strain G1 (GenBank LC476648 and MT318687) and 6 sheep, 4 goats and 2 human samples was 99.9% identity with LC476648 and MT318687 due to slight variation was observed in position 58 (T→C). After that, when they were aligned with current Iranian and Turkish G1 sequences, they had higher percent identities of 99.9–100%. Results of the present works represented that, most of the isolates analyzed belonged to the G1 genotyping (sheep strain), and shown that this strain is actively transmitted throughout the Kurdistan region and surrounding countries [21-23], and the sheep play a significant role in human transmission cycle and the and the absence of G4, G6, and G7 genotype in this investigation is probably explained by the fact that in Kurdistan region, these genotype are specific to the species that were not slaughtered in slaughter house, like baffle, horse and pigs. The result disagreement with [24], who observed that in Iraq, the majority of CE patients (60) were infected with hydatid cysts of either G4 (43٪), G5 (20٪), and G6/G7 (7٪) and [25] reported that, 38 isolates were sequenced by using polymerase chain reaction to amplify mitochondrial COX1 gene in the south-east of Iran and The genotype G1 was most prevalent in sheep (86.7%), cattle (80%), camels (44.4%), and

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goats (100%). Furthermore, [26] revealed that, of the 20 goat samples tested, 17 isolates (85%) were of the sheep strain's G1 genotype and the other three isolates (15%) were of the G6 genotype.

5- Conclusion

The multiplex PCR assay is benefit for differential diagnosis, molecular characterization and epidemiological survey of *Echinococcus spp*. and *Taenia spp*. The current study's genotyping data strongly show that the majority of the animal and human isolates found in Erbil Provence are of the G1 and G1BC genotype. In order to properly condemn their impacted offal and conduct further genetic investigation, we propose developing new methodologies. These safety measures will be very helpful in shortening the life cycle of the *Echinococcus spp.* and managing the illness.

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8- Author contribution: Authors contributed equally in the study.

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