Molecular analysis of *Echinococcus granulosus*: A comparison between cattle and sheep isolates in Babylon province, Iraq

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Abstract

Cystic echinococcosis (CE) is the metacestode parasite Echinococcus granulosus, a serious health and economic issue in many regions of the world. The study aimed to determine the genotypes and phylogenetic relationships of *E. granulosus* isolated from cattle and sheep using PCR-based genotyping of the mitochondrial cytochrome C oxidase subunit 1 (COX1) gene. Additionally, it identified the genetic diversity of *E. granulosus* circulating in various regions of Babylon province, Iraq. 112 liver hydatid cyst samples were collected from (52) slaughtered cattle and (60) sheep. The phenol-chloroform technique was used to extract hydatid cyst DNA, which was amplified by PCR using Cox1 gene-specific primers. Sequence analysis was conducted on 20 positive samples from both hosts using Multiple Sequence Alignment with ClustalW compared to Gen-Bank sequences. The UPGMA method was utilized in MEGA 11 to create a phylogenetic tree and evaluate its reliability. Out of the 20 sequenced samples, the sequencing results indicated that 15 samples belonged to the predominant G1 strain, two to the G2 strain, and three to the G3 strain, which were detected in both cattle and sheep. The predominant strain of hydatid cysts is the G1 strain in both cattle and sheep, which shows the important role of this genotype in the spread of the parasite in this region. The first detection of genotype G2 in cattle and sheep indicates genetic diversity and the spread of infection in different hosts. Subsequently, the results can be applied to lessen human infections and interfere with the parasite's life cycle.

Introduction

Cystic echinococcosis (CE) is a chronic parasitic zoonotic disease caused by larval-stage infection with the tapeworm (*Echinococcus granulosus*) (1,2). The disease considerably impacts human and animal health and causes significant economic losses in the endemic area (3-5). The domestic life cycle of this parasite is complex, with the larval stage (hydatid cysts) occurring in most organs of intermediate hosts, such as the liver, lungs, spleen, brain, and bones. At the same time, adult worms inhabit the small intestine of the canine, which serves as the final host (6). The

E. granulosus has different host affinities, and mitochondrial DNA sequences have identified 10 distinct genetic types G1 to G10. These include G1, G2 sheep strains, G3, G5 buffalo strains, G4 horse strains, G6 camel strains, G7, G9 pig strains, and G8, G10 deer strains (7). Cattle and sheep are primary intermediate hosts for this parasite, with genotypes G1 and G3 being the most prevalent causes of hydatidosis (8,9). The genetic variety of this parasite may cause distinct genotypes to behave differently in terms of pathogenicity, host specialization, treatment sensitivity, antigenic potency, and life cycle and transmission methods (2,10). Hydatidosis is highly endemic in Iraq and has a significant impact on both

human health and livestock productivity (11). Several previous studies conducted in Iraq have demonstrated notable variation in prevalence among various livestock, such as cattle (4/720), sheep (96/4800), and goats (5/960) in Mosul (12). A study recorded a prevalence of 3.12% in cattle in Kirkuk province (13), while 36.15% in sheep in Basra city (14). In Mosul recorded 7.29 and 5.45% in sheep and goats respectively 915). Ranchers in affected communities endure significant financial losses due to hydatid cysts, along with health issues and other clinical complications in infected individuals (15).

The study aimed to determine the genotypes and phylogenetic relationships of *E. granulosus* isolated from cattle and sheep, using PCR-based genotyping of the mitochondrial cytochrome C oxidase subunit 1 (COX1) gene. Additionally, it aimed to identify the genetic diversity of *E. granulosus* genotypes circulating in various regions of Babylon province, Iraq.

Materials and methods

Ethical approval

It was obtained following the guidance of the Middle Technical University Medical Ethics Committee in Al Za'franiya, Baghdad, P.C.: 10074 (ECR No: 31, Approval Date 24/10/2024), which aligns with the relevant Iraqi legislation.

Samples collection

This study involved the collection of 112 cysts from 52 cattle and 60 sheep at slaughterhouses in Babylon province, which took place from March 2024 to September 2024. The samples were cleaned thoroughly with water before the hydatid cysts were removed from the slaughtered cattle and sheep and placed in an ice-cooled container before being transported to the lab. First, 70% ethanol was used to sterilize the surface of the hydatid cysts. The cyst fluid was extracted using sterile 10-ml medical syringes, and the protoscolices were collected in a sterile environment (16). The protoscolices and cyst fluid were removed. The germinative layer was taken out and placed in a sterile petri-dish containing 0.9% physiological saline solution to extract the maximum number of protoscolices. The layer was then washed repeatedly with phosphate buffer solution (PBS) in a washing bottle, placed in sterile tubes, and subjected to centrifugation three times at 3000 rev/min for 15 minutes. These tubes were preserved with fragments of the germinal layer by adding 70% ethyl alcohol, which was later used in genetic sequencing and polymerase chain reaction (PCR) to identify the prevalent parasite strains (17).

DNA extraction

According to the purification kit's instructions, DNA was isolated from hydatid cysts and subsequently frozen for later use. Genomic DNA was extracted from the frozen samples using the DNA micro kit produced by Geneaid in the United States. As the manufacturer directed, proteinase K was used to lyse the cells during extraction. The extracted gDNA was stored at -20°C in a refrigerator until it was required for PCR amplification.

PCR (polymerase chain reaction)

The Mitochondrial Cytochrome Cox1 gene was amplified by PCR using a primer that facilitated the detection and genotyping of *E. granulosus*. The table 1 details how these primers were designed and supplied by Macrogen Company, Korea (18).

Table 1: Primer based on the mitochondrial cytochrome c oxidase 1 gene

Primer		Sequence	Amplicon
Cox1 gene	F	TTTTTTGGGCATCCTGAGGTTTAT	450bp
	R	TAAAGAAAGAACATAATGAAA ATG	

The Maxime PCR PreMix Kit was used to prepare the PCR master mix, which was then completed according to the following business instructions: 5-50 ng (5µl) of DNA template PCR Water 13µl, forward primer (10 pmol) 1µl, reverse primer (10 pmol) 1µl, and a total volume of 20 µl. The reaction is denatured for five minutes at 94°C initially, followed by thirty cycles consisting of 45-second denaturation at 94°C, 45-second annealing at 50°C, and 45-second extension at 72°C. This is followed by a final 7-minute extension at 72°C and permanent holding at 4°C. The PCR products were assessed using 1% agarose gel electrophoresis and UV light-induced ethidium bromide staining (19).

Sequence analyses

Multiple sequence alignment analyses of the incomplete kDNA gene relied on DNA sequencing data obtained through Molecular Evolutionary Genetics Analyses version 11 (Mega 11). This included an assessment of evolutionary distances using the ClustalW alignment with the UPGMA technique, along with the composite likelihood method (20).

Statistical analyses

The Chi-square (x^2) test was used in statistical investigations to determine whether variables were calculated based on maximum independence. Values less than or equal to 0.05 were deemed statistically significant by SPSS statistical software, version 31.0 (21).

Results

Fertility of hydatid cysts

In examined cattle, 52 liver hydatid cysts were found. Of these, 18 (34.6%) were fertile and filled with viable protoscolices, while 34 (65.4%) were sterile (Figure 1). In sheep, 60 liver hydatid cysts were found: 21 (35%) were fertile and filled with viable protoscolices, while 39 (65%) were sterile (Figure 2 and 3).

Figure 1: Fertile and sterile hydatid cysts in the livers of cattle.

Figure 2: Hydatid cysts from sheep liver.

Molecular diagnosis

The agarose gel electrophoresis results showed distinct bands at 450 bp for *E. granulosus* in cattle and sheep hydatid cysts (Figures 4 and 5), respectively. 112 samples of hepatic hydatid cysts were collected from various slaughterhouses in Babylon province (52 cattle samples and 60 sheep samples). PCR was used to amplify samples with Cox1 gene-specific primers.

Figure 3: Viable protoscolices at 10X.

Figure 4: Agarose gel electrophoresis at 1% demonstrating the PCR products of the mitochondrial cox1 gene in *E. granulosus* from bovine infection samples, including hydatid cysts. Ethidium bromide is utilized to stain the gel. The bands in lane M represent the DNA markers, which are used to estimate the sizes of the PCR products in the other lanes. The bands in lanes 1-10 show the PCR products of the mitochondrial cox1 gene. Each band measures approximately 450 bp, which aligns with the expected size of the PCR results.

Figure 5: Agarose gel electrophoresis at 1% demonstrates the PCR products of the mitochondrial cox1 gene in *E. granulosus* from sheep infection samples, including hydatid cysts. Ethidium bromide is employed to stain the gel. The bands in lane M represent the DNA markers used to estimate the size of the PCR products in the other lanes. The bands in lanes 1-10 are the PCR products of the mitochondrial cox1 gene. Each band measures approximately 450 bp, which is consistent with the expected size of the PCR results.

DNA Sequence

The mitochondrial cytochrome Cox1 gene was used in the DNA sequencing process to conduct a genetic genotyping investigation of *E. granulosus* in local isolates of cattle and sheep. The nucleotide sequences of the mitochondrial Cox1 gene from the cattle and sheep isolates exhibited genetic variation (substitution mutation) and similarity (*) as indicated by the results of a multiple sequence alignment analysis between the local *E. granulosus* isolates and related genotypes from NCBI-BLAST, using ClustalW (Figures 6 and 7). The local cattle isolates (PQ157654- PQ157656 and PQ157658- PQ157662) were closely related to the NCBI-BLAST *E. granulosus* isolate G1 (MN732819.1), except for PQ157657, which clustered with G2 (KC109660.1), and PQ157663, which clustered with G3 (MW421883.1). The genetic distances between these isolates ranged from 0.01 to 0.04, indicating varying degrees of genetic divergence (Figure 8). The genetic homology sequence identity varied between 99.23% and 99.56% among the local cattle isolates and related *E. granulosus* strains from NCBI-GenBank (Table 2).
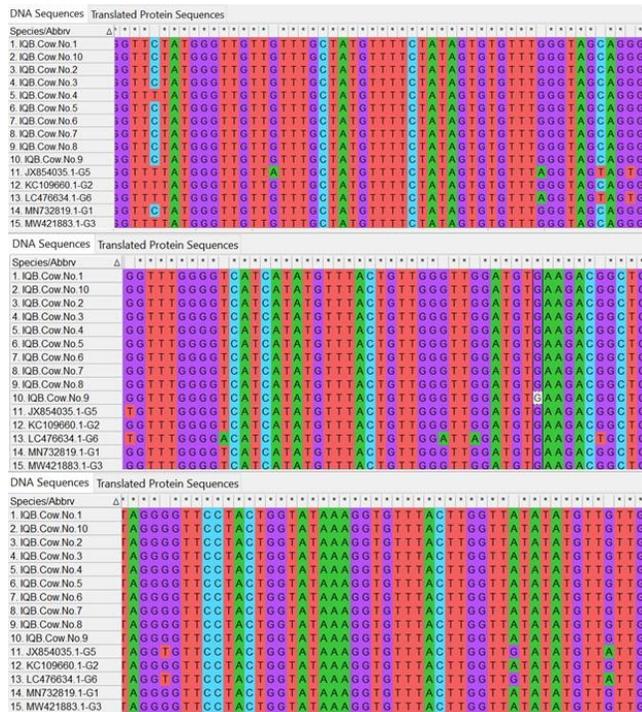

Figure 6: The partial sequence of the mitochondrial Cox1 gene underwent multiple sequence alignment analysis using the CLUSTALW method on local isolates of *E. granulosus* from cows utilizing NCBI-BLAST associated genotypes of *E. granulosus* isolates. The multiple alignment analysis revealed genetic variance (substitution mutation) and similarity (*) in cow isolates' mitochondrial Cox1 gene nucleotide sequences.
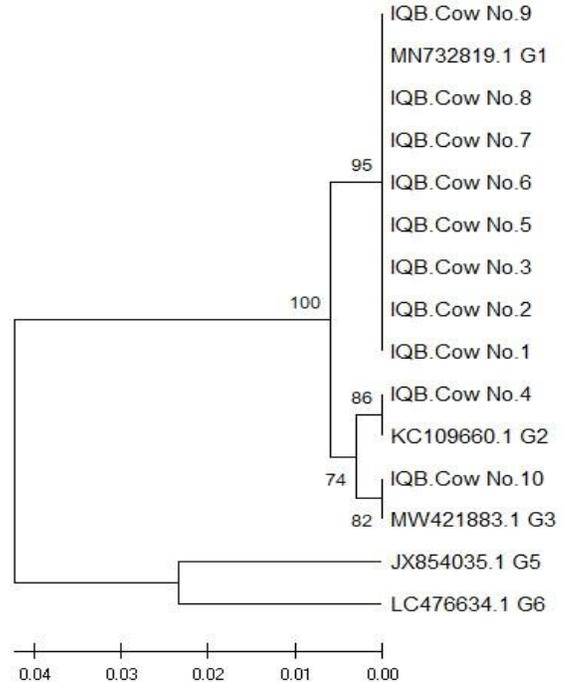

Figure 7: *Echinococcus granulosus* genotypes were identified through phylogenetic tree analysis based on the partial sequence of the mitochondrial Cox1 gene. The phylogenetic tree, constructed using partial mitochondrial Cox1 gene sequences and the UPGMA method in MEGA 11, examines the genetic relationships of *E. granulosus* isolates. Ten local cow isolates (PQ157654- PQ157656 and PQ157658- PQ157662) were closely related to the NCBI-Blast *E. granulosus* isolate G1 (MN732819.1), except for PQ157657, which clustered with G2 (KC109660.1) and PQ157663, which clustered with G3 (MW421883.1). These isolates exhibit genetic distances ranging from 0.01 to 0.04 between them, indicating different levels of genetic divergence.

The local sheep isolates of *E. granulosus* were submitted to the NCBI GenBank and identified by accession numbers (PQ157664- PQ157673). The phylogenetic tree analysis of genetic relationships showed that local sheep isolates (PQ157666- PQ157671 and PQ157673) are closely related to the NCBI-BLAST *E. granulosus* isolate G1 (MN732819.1). The PQ157672 clustered with G2 (KC109660.1), while the PQ157664 and PQ157665 clustered with G3 (MW421883.1). The genetic distances between these isolates ranged from 0.01 to 0.04, indicating varying degrees of genetic divergence (Figure 9). The homology sequence identity between the local sheep isolates and related *E. granulosus* strains in NCBI-GenBank demonstrated a genetic homology sequence identity ranging from 99.23 to 99.70% (Table 3).
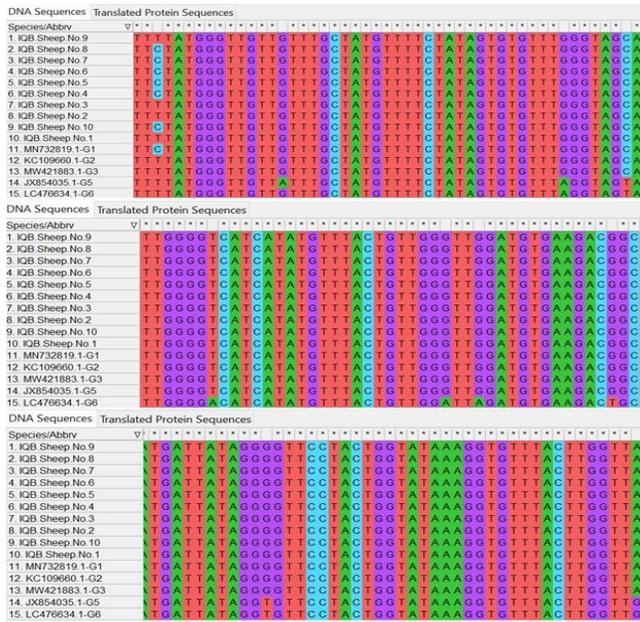
Figure 8: The partial sequence of the mitochondrial Cox1 gene was analyzed through multiple sequence alignment using CLUSTALW to compare local isolates of *E. granulosus* from sheep with isolates of related genotypes of *E. granulosus* obtained from NCBI-BLAST. The results of the multiple alignment analysis demonstrated genetic variance (substitution mutation) and similarity (*) between nucleotide sequences of the mitochondrial Cox1 gene from the sheep isolates.

Genetic diversity over sequence pairs between different genotypes of *E. granulosus* in cattle and sheep

The averages of all sequence pairs depend on the number of base differences per site between cattle and sheep genotypes. This analysis included 23 nucleotide sequences. All sequence positions containing missing data and gaps were completely eliminated. The final dataset detected 266 positions. The evolutionary analyses were conducted in MEGA11 (Table 4).
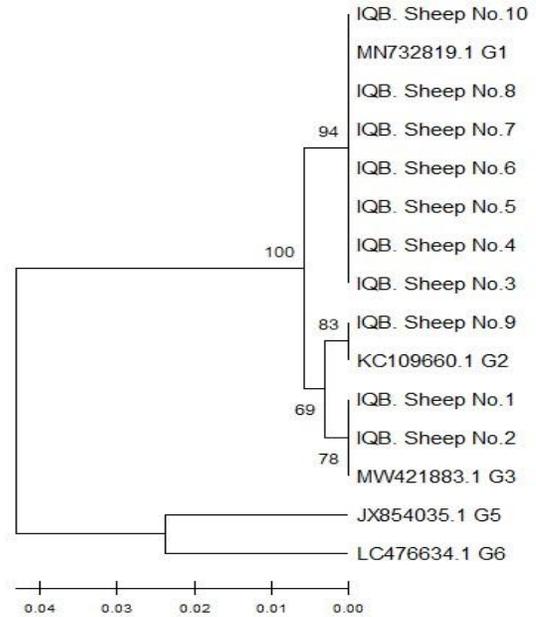

Figure 9: *Echinococcus granulosus* genotypes were identified through phylogenetic tree analysis based on the partial sequence of the mitochondrial Cox1 gene. The phylogenetic tree, constructed using partial mitochondrial Cox1 gene sequences and the UPGMA method in MEGA 11, evaluates the genetic relationships among *E. granulosus* isolates. Ten local sheep isolates (PQ157666- PQ157671 and PQ157673) were closely associated with the NCBI-Blast *E. granulosus* isolate G1 (MN732819.1), PQ157672, which clustered with G2 (KC109660.1), as well as PQ157664 and PQ157665, clustered with G3 (MW421883.1). These isolates display genetic distances ranging from 0.01 to 0.04, suggesting varying levels of genetic divergence.

Table 2: Local *Echinococcus granulosus* cow isolates and NCBI-BLAST isolates of *Echinococcus granulosus*-related genotypes exhibit the same homology sequence identity

Local isolate	Genbank submission accession number	NCBI-BLAST Homology Sequence identity		
		NCBI BLAST identity Genotype	Accession number	Identity (100%)
IQB.Cow No.1	PQ157654	Genotype 1	MN732819.1	99.33%
IQB.Cow No.2	PQ157655	Genotype 1	MN732819.1	99.43%
IQB.Cow No.3	PQ157656	Genotype 1	MN732819.1	99.56%
IQB.Cow No.4	PQ157657	Genotype 2	KC109660.1	99.33%
IQB.Cow No.5	PQ157658	Genotype 1	MN732819.1	99.45%
IQB.Cow No.6	PQ157659	Genotype 1	MN732819.1	99.34%
IQB.Cow No.7	PQ157660	Genotype 1	MN732819.1	99.23%
IQB.Cow No.8	PQ157661	Genotype 1	MN732819.1	99.34%
IQB.Cow No.9	PQ157662	Genotype 1	MN732819.1	99.34%
IQB.Cow No.10	PQ157663	Genotype 3	MW421883.1	99.56%

Table 3: Local *Echinococcus granulosus* sheep isolates and NCBI-BLAST isolates of *Echinococcus granulosus*-related genotypes exhibit the same sequence homology identity

Local isolate	Genbank submission accession number	NCBI-BLAST Homology Sequence identity		
		NCBI BLAST identity Genotype	Accession number	Identity (100%)
IQB. Sheep No.1	PQ157664	Genotype 3	MW421883.1	99.60%
IQB. Sheep No.2	PQ157665	Genotype 3	MW421883.1	99.45%
IQB. Sheep No.3	PQ157666	Genotype 1	MN732819.1	99.55%
IQB. Sheep No.4	PQ157667	Genotype 1	MN732819.1	99.53%
IQB. Sheep No.5	PQ157668	Genotype 1	MN732819.1	99.70%
IQB. Sheep No.6	PQ157669	Genotype 1	MN732819.1	99.36%
IQB. Sheep No.7	PQ157670	Genotype 1	MN732819.1	99.23%
IQB. Sheep No.8	PQ157671	Genotype 1	MN732819.1	99.34%
IQB. Sheep No.9	PQ157672	Genotype 2	KC109660.1	99.36%
IQB. Sheep No.10	PQ157673	Genotype 1	MN732819.1	99.59%

Table 4: Genetic diversity across sequence pairs among various genotypes of *E. granulosus* in cattle and sheep

Genetic diversity	G1 cattle	G2 cattle	G3 cattle	G3 sheep	G2 sheep	G1 sheep
G1 cattle						
G2 cattle	0.0112782					
G3 cattle	0.0075188	0.0037594				
G3 sheep	0.0075188	0.0037594	0.0000000			
G2 sheep	0.0100251	0.0012531	0.0025063	0.0025063		
G1 sheep	0.0000000	0.0112782	0.0075188	0.0075188	0.0100251	

Discussion

Genotyping *E. granulosus* is the initial step in identifying the parasite, managing its pathogenicity, and decreasing infection by determining genotypes. The agarose gel electrophoresis results 1% revealed distinct bands at 450 bp for *E. granulosus* in the cattle and sheep hydatid cysts, using specific primers for the cox1 gene amplified by PCR. These results align with previous studies (18,22) that indicated the cox1 gene's size as 450 bp. Most stray dogs in these areas consume the intestines of domestic animals infected with hydatid cyst larvae. This condition may be enough to worsen the endemic situation.

The molecular characterization of *Taeniidae* tapeworms, particularly at the Cox1 and Nad1 loci, has been successfully accomplished through mitochondrial DNA (mtDNA) sequencing (23). The evolutionary change rate of the mitochondrial Cox1 gene is rapid enough to distinguish between distinct species and slow enough to analyze variation within the same species, making it the ideal gene for uncovering genetic variation. Therefore, the mitochondrial Cox1 gene was utilized in the current study to generate DNA barcodes and differentiate between various helminth species (24).

The results showed that, depending on Cox1 gene sequence analysis, three genotypes existed in sheep, and two genotypes existed in cattle in Babylon province: sheep strains (G1 and G2) and buffalo strains (G3). This was the first report of genotype G2 in sheep and cattle in Iraq. The most predominant genotype infecting cattle was G1 (90%), while the least prevalent genotype was G2 (10%). These findings align with several previous studies conducted in various regions of Iraq (25-29), but contradict the results which reported a 100% incidence rate of calf infections from G3 in Al-Diwaniyah and Al-Najaf governorates (21).

In sheep, the most predominant genotype was the G1 sheep strain (60%), followed by G3 (30%), with the lowest genotype being G2 (10%). This study aligns with a study from Palestine (30), which showed that sheep were infected with genotypes G1 (66.7%), G2 (19.0%), and G3 (14.3%), respectively. Additionally, a study in Al-Diwaniyah province found, by analyzing five samples, that G1 and G3 are common in infections with hydatid cysts, and the genotype G1 is more prevalent than genotype G3 (31). It also supports a study conducted in northern Iraq (25), which detected that the G1 strain, followed by the G3 strain, is the most prevalent in sheep infected with hydatid cysts.

These results align with several previous studies conducted in various regions of Iraq (25-33). Babaei *et al.* (34) demonstrated that G1 is the sole genotype or dominant genotype of *E. granulosus* in Bushehr, Iran. Irehan *et al.* (35) identified *E. granulosus* G1 and G3 in Turkey. At the same time, in Saudi Arabia, Alkhaldi (36) indicated that all isolates of hydatid cysts from sheep (GenBank accession numbers OQ970593-OQ970597) exhibited strong sequence similarity with the G1 isolate of *E. granulosus* sourced from sheep. The genetic diversity of *E. granulosus* is a complex and multifaceted topic. In domesticated animals such as sheep,

goats, cattle, and camels, the genetic diversity of *E. granulosus* has been studied in various regions, including Iraq, Iran, Turkey, and China.

In Iraq, studies indicated that high-diversity haplotypes were recorded (17 haplotypes). However, the dominant G1 (95%) and G3 (5%) genotypes were responsible for the infection with echinococcosis (29). In Iran, studies found that the G1 genotype was dominant in infected intermediate hosts (37,38). Another study in Iran found that the G1-G3 complex genotype was present in all sheep, goat, cattle, and buffalo isolates. In contrast, the G2 genotype was present in only one isolate (39). In Turkey, a study found that the G1 genotype was present in 78.9% of the isolates, while the G3 genotype was present in 5.3% of the isolates; it also found that the G1/G3 complex genotype was present in 15.8% of both sheep and cattle isolates (40). In China, a study found that the G1 genotype was present in all sheep and cattle isolates and that the G1-G3 complex genotype was the dominant genotype in the region (41).

Overall, the studies indicate that the genetic diversity of *E. granulosus* in domestic animals is intricate and multifaceted, featuring multiple genotypes across various regions. It's important to highlight that the genetic diversity of *E. granulosus* can impact the diagnosis, treatment, and control of cystic echinococcosis. Thus, ongoing research into the genetic diversity of *E. granulosus* is essential for enhancing our understanding of this complex parasite.

The differences in the results may stem from variations in host breeds, study areas, feeding practices, grazing, and the customs and traditions of the local inhabitants. Changes observed in the samples are influenced by environmental conditions, temperature, and the presence of final hosts in these regions. Consequently, this study demonstrated that sequence analysis effectively identifies and categorizes the species involved. It is also straightforward, quick, and highly reliable. Furthermore, for genetic resource management and enhanced efficiency, it provided a wealth of valuable genetic information about the species in question (42).

Conclusions

This study's results offer important information about the molecular epidemiology of *E. granulosus* in livestock in Babylon province, Iraq. The range of genotypes G1, G2, and G3 found in the sheep and cattle populations in the region indicates the parasite's genetic diversity. This study emphasizes the importance of ongoing surveillance and molecular characterization of *E. granulosus* to guide focused control and prevention initiatives in endemic regions.

Acknowledgment

Conflict of interest

There is no conflict of interest.

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التحليل الجزيئي لطفيلي المشوكة الحبيبية: دراسة مقارنة لعزلات الأكياس المائية في الأبقار والضأن في محافظة بابل، العراق

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الخلاصة

الطور اليرقي لطفيلي المشوكة الحبيبية هو المسبب لداء الاكياس المائية، وله تأثيرات صحية واقتصادية خطيرة في العديد من مناطق العالم. الهدف من الدراسة هو تحديد الأنماط الجينية والعلاقات التطورية لطفيلي المشوكة الحبيبية المعزول من الأبقار والضأن باستخدام النمط الجيني القائم على تفاعل البلمرة المتسلسل لجين وحدة أوكسيديز السيتوكروم سي ١ للميتوكوندريا، بالإضافة إلى تحديد التنوع الجيني لطفيلي المشوكة الحبيبية المستوطن في مناطق مختلفة من محافظة بابل، العراق. تم جمع ١١٢ عينة من الكيس المائي الكبدي من ٥٢ الأبقار المذبوحة و ٦٠ من الضأن المذبوحة. استخدمت تقنية الفينول-الكلوروفورم لاستخراج الحمض النووي للكيس المائي الذي تم تضخيمه

بواسطة تفاعل البوليميرات المتسلسل باستخدام بادئات خاصة بجين وحدة أوكسيديز السيتوكروم سي ١. تم الوقوف على تسلسل عشرون عينة موجبة من كلا المضيفين وتحليلها باستخدام محاذاة التسلسل المتعدد بواسطة Clustal W، ومقارنتها بتسلسلات بنك الجينات وباستخدام طريقة UPGMA في MEGA 11 لتحديد شجرة النشوء والتطور وتقييم موثوقيتها. من بين ٢٠ عينة متسلسلة، أظهرت نتائج التسلسل أن ١٥ عينة متسلسلة تنتمي إلى السلالة السائدة الأولى، وعينتان تنتميان إلى

السلالة الثانية و٣ عينات تنتمي إلى السلالة الثالثة التي اكتشفت في كل من الأبقار والضأن. السلالة السائدة من الكيس المائي هي السلالة الأولى في كل من الأبقار والضأن، وبالتالي يظهر الدور المهم لهذا النمط الجيني في انتشار الطفيلي في هذه المنطقة والكشف الأول عن النمط الجيني الثاني في كل من الأبقار والضأن يعمل كمؤشر للتنوع الجيني وانتشار العدوى في مضائف مختلفة، وبالتالي يمكن تطبيق النتائج لتقليل الإصابات البشرية والتدخل في دورة حياة الطفيلي.