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Molecular characterization study of Hydatid cysts that isolated from sheep carcasses at holy Kerbala city

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***Abstract*— Hydatid cysts are the immature form of the tapeworm Echinococcus granulosus, responsible for causing a zoonotic disease known as echinococcosis, sometimes known as hydatid disease. Ten different genotypes of this parasite have been formally identified. The present study aimed to sequence and characterize the genotypes of E. granulosus using the antigen B subunit 2 gene of isolates obtained from sheep in Karbala Governorate, Iraq. Samples were collected between October 2024 and May 2025, and included sheep of both sexes and younger than five years of age. These samples were obtained from the Karbala slaughterhouse and from sheep farmers in the Al-Hussainiya area. A total of 5547 sheep were examined through necropsy. Hydatid cysts were detected and recorded in several organs, distributed as follows: 2635 liver samples, 1470 lung samples, 664 kidney samples, 212 mesenteric samples, and 549 meat samples. The samples were transported to the laboratory using sterile containers to ensure their safety. Molecular analysis of Echinococcus granulosus isolates from sheep in Kerbala provided important insights into the parasite's genetic diversity and local prevalence. Mitochondrial gene sequencing, particularly of CO1 and ND1, was used to identify genotypes and compare them to global reference strains, enabling a precise genetic map of the parasite isolates in the region.**

The study aim was to identify and characterize the genotypes of Echinococcus granulosus isolated from sheep in Karbala Governorate, Iraq, using molecular analysis of the antigen B subunit 2, CO1, and ND1 genes to assess genetic diversity and local distribution.

***Keywords* — Hydatid cysts, Echinococcus granulosus, Phylogenetic tree, Meat, PCR.**

INTRODUCTION

Hydatid illness, another name for echinococcosis, is a communicable illness that causes a significant continuous incidence of life years adjusted for disabilities (DALYs) worldwide (1). All nations in the Middle East and the western Mediterranean area are endemic for echinococcosis (2). The disease known as the infection is caused by the larval phase of the worm *E. granulosus*, species belongs to the phylum the genera Platy. Intermediate hosts, such as domestic animals like sheep and goats (called pastoral hydatidosis) especially wild animals like moose, the reindeer, and caribou (called sylvan hydatidosis), can contract the infection.

Inadvertent ingesting water or food that is tainted through the parasite's egg can expose individuals to an *E. granulosus* illness. The waste products of afflicted animals contain these eggs. This parasite's anomalous intermediary hosts were people. The organ known as the liver is where hydatid cysts most frequently form in people, occurring in fifty percent to ninety-three of instances (3). The juvenile worm stage, which is the mature version of *E. granulosus*, lives in the digestive tract of dogs. Intestinal tapeworm's eggs discharge larvae enter the intestines once they are eaten by intermediary hosts, including people, rats, antelope, cattle, and various other animals. The resulting embryos are then put into circulation through the portal. Although some of the fertilized embryos are trapped within the liver, the remainder pass through that organ and spread to other organs, and eventually develop as cysts of hydatid tissue (4). The organ responsible for liver function was impacted in 70% to 75% of cases with echinococcosis with cysts (CE), whereas the respiratory tract were impacted in between ten and twenty percent of cases. Nonetheless, cysts of hydatid tissue can form in every bodily organ (5). The mature worm has three to four proglottids and is small, averaging 3 to 6 mm in length and 0.5 mm in width. With differences in the physical characteristics of mature organisms, the capacity to

infect particular hosts, and the severity of sickness, it is clear that While CE may appear in all organs and tissues of humans and other intermediate host animals, the liver generally accounts for the bulk of infections, with the respiratory tract coming in second (6). *E. granulosus, E. vogeli, E. oligarthrus, E. shiquicus, and E. felidis* are the six types species Echinococcus tapeworms that have been identified. Two species of significant medical importance are Echinococcosis (CE) produced by *E. granulosus* and AE caused by E. *multilocularis*. (AE) (7). Serological investigation is usually used to diagnose hydatid illness. This procedure and indirectly the formation of he (IHA) are two popular procedures. The IHA testing offers an accuracy range of from ninety percent to along with an accuracy of roughly 60% for lung or calcification tumors and eighty-eight overall liver or intraperitoneal illness (8). There are various classifications for E. granulosus. Secretory and attached to the membrane (S/M) enzymes are essential for evading the body's immune response following infestation through the pathogen's larval development. A material that triggers the body's immunological reaction is known as an antigens (9). A thermostable glycoprotein having a relative molecular weight of 160 kDa is the natural Antigen B (AgB). Whenever examined using continuous gel electrophoresis, native AgB generates three separate subunits under decreasing circumstances (Maddison et al., 1989). One important E. *granulosus* secreted antibody used for the identification of hydatid illness is antigen B subunit 2 (AgB2). An essential diagnostics tool, AgB2 is a member of the AgB protein class, is strongly immunoreactive, and plays a crucial role in the parasite's immunological escapes. This antigenic properties is extremely highly specific and sensitive, eliciting precise antibody responses in infected hosts as shown by immunological techniques like ELISAs. Atomically speaking, AgB2 is the gene that is utilized in PCR-based testing to look for *E. granulosus* DNA in environmental or clinical samples. It is also the only gene required to confirm infection, particularly in areas where infection is prevalent. AgB2 immune regulation not only aids in diagnosis but also raises the possibility of whether it might be a viable treatment and vaccine option. They also improved diagnosis and control methods and validated the significance of Agb, which stands2 in the investigation of hydatid illness. (10, 11).

Phylogenetic tree of *E .granulosus*

Genetic analysis using the cytochrome c oxidase subunit 1 (COX1) gene revealed that the local Iraqi strain PVO52631.1 falls within a distinct genetic cluster that forms an independent branch in the genetic tree, indicating a close evolutionary relationship with several global strains. The genetic tree showed that this strain shares a single evolutionary node with strain MZ714551.1, isolated from Cluster_19_Cox38, strain MF002477.1, isolated from KU05, as well as MN678533.1 and MG608308.1, representing the EgAg2 genotype. This indicates that all of these strains descend from a common ancestor, forming a monophyletic clade with clear evolutionary significance. This genetic correlation suggests that the Iraqi strain is not evolutionarily separate from some global strains, but may have derived from the same geographic source or evolved within a similar environmental context. This is consistent with (Bowles et al., 1992 and Thompson & McManus, 2002), who confirmed the global distribution of *Echinococcus granulosus* strains with common origins, particularly between the Middle East and Central Asia. In a similar vein, Nakao et al., 2010, noted the importance of mitochondrial genes, particularly COX1, in differentiating genotypes within the genus Echinococcus. This is supported by our results, which demonstrate a close relationship between the Iraqi strain and some global reference strains, reinforcing the hypothesis of overlapping geographic distribution or historical transmission of strains between countries. Thus, the results of the genetic tree do not place the Iraqi strain in an isolated evolutionary position, but rather confirm its belonging to a homogeneous evolutionary group containing strains of known origin and taxonomy, providing strong evolutionary evidence that can be used to trace the parasite's lineages and the history of its geographical spread (12).

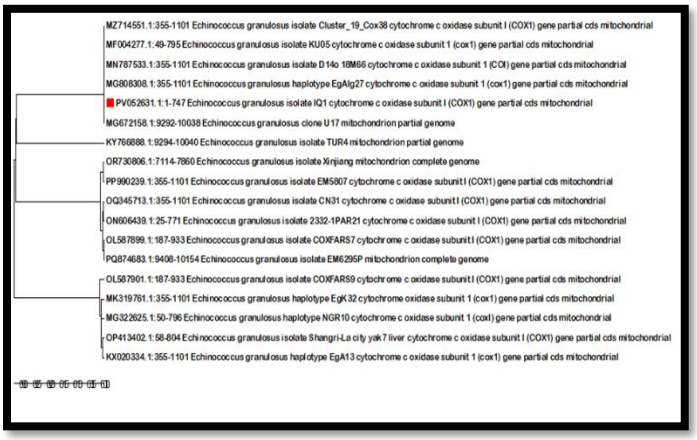

Figure 1. phylogenetic tree of *E. granulosus* strains

The Iraqi local strain PVO52631.1 clearly occupies an evolutionary position within a group of other global strains, indicating its genetic closeness to these strains. The Iraqi sample was found to be closely related to strains such as MZ714551.1 (Cluster_19_Cox38), MF002477.1 (KU05), and MN678533.1 (D140), in addition to MG608308.1, which represents the genotype known as EgAg2. This genetic cluster forms a monophyletic group, meaning that these strains share a common evolutionary ancestor without the intrusion of strains from outside groups. This pattern of genetic relationship reflects the possibility of a common ancestry between the local Iraqi strain and some strains distributed in Central Asia and parts of China, especially since some of these samples, such as D140 and KU05, have previously been documented in previous studies as representatives of Asian lineages (13). The positioning of the Iraqi strain within this clade supports the hypothesis of geographic exchange or animal migrations that may have contributed to the transmission of parasites between

multiple regions throughout history, whether through trade channels or through the movement of livestock.

Materials and Methods

Samples collection (from Liver, Lung, Kidney, Mesentery and Meat) were collected from sheep under 5 years old from both sexes suffering from kerbala city abattoir and Al-Hussenyia sheep farmers in Kerbala during the period from October 2024 to May 2025. A total number 5547 of sheep was examined via autopsy for hydatid cysts in slaughterhouses 2635 samples from liver , 1470 form lungs , 664 from kidney , 212 Mesentery and 549 Meat (figure 2). Samples were transported in sterile container to the laboratory.

DNA extraction and PCR DNA extraction from samples (i.e. lesion and tissue samples) was conducted using the Mini BEST Universal Genomic DNA Extraction Kit Ver.5.0 (TaKaRa) according to the manufacturer's instructions. To identify Echinococcus spp., we used four primer pairs to perform parallel PCR tests of each sample. A Taeniidae family universal primer pair CO1JP2 (F/COI and R/COI, Table 1) (12) was used to amplify a region of approximately 874 bp in length of the mitochondrial cox1 gene. Three species-specific nad1 gene primer pairs (ND1Eg, ND1Em and ND1Es) were used to detect *E. granulosus* (s.s.) respectively (Table 1) [13]. All PCRs were performed in 50 µl volumes with 4 µl template DNA, 1 µl of the primers (10 µmol/l), 1 µl of bovine serum albumin (BSA, TaKaRa, Dalian, China), and 25 µl Premix Taq (Ex Taq Version 2.0 plus dye, TaKaRa), made up to 50 µl with deionized H2O (dH2O). PCR of CO1JP2 comprised 30 cycles of 30 s at 94 °C, 45 s at 52 °C, 90 s at 72 °C, and a final extension step of 72 °C for 5 min. Parameters of the PCRs for the three nad1 species specific primer pairs were: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 45 s at the annealing temperature of each primer pair (Table 1), 72 °C for 90 s, and then 72 °C for 10 min. All PCRs were run on a DNA thermal cycler (Bio-Rad, Hercules, CA, USA).(14)

Table 1. Primer sequences, lengths of PCR amplicons and annealing temperatures

Primer Name	Forward Primer	Species	Target Gene	Amplicon Length (bp)	Annealing Temp (°C)	Reference
CO1JP2 Forward	TTGAATTTGCCACGTTTGAATGC	*Taeniidae gen. sp.*	cox1	875	52	[26]
CO1JP2 Reverse	GAACCTAACGACATAACATAATGA					
ND1Eg Forward	GTTTTTGGCTGCCGCCAGAAC	*E. granulosus*	nad1	226	62	[27]
ND1Eg Reverse	AATTAATGGAAATAATAACAAACTTAATCAACAAT					

Laboratory protocol The following reagent was added for each tube on ice .Green master mix 25 µl Upstream primer 2.5 µl Downstream primer 2.5 µl(a light overtaxing was used) The mixture was mixed thoroughly by shaking & spin .Five µl of DNA sample was added by using specified pipette for sampling of DNA .Complete the volume to50µl with nuclease-free water, mix for 3-5 seconds. One drop (20-25µl) of mineral oil was added to each tube. After getting the thermo cycler hot, we added the PCR tubes and began the reaction ."The comb was placed at one end of the casting tray after the agarose 2% gel was assembled ,the gel was then poured into the tray and let to cool for 30 minutes at room temperature, Once the comb was removed, the electrophoresis chamber was reassembled with gel". TBE-electrophoresis buffer was poured into the chamber until it was between 3 and 5 millimeters(mm) above the gel's surface (15)

"Loading and running DNA in agarose gel" The 2% agarose gel had DNA (10 µl) and bromophenol blue (3 µl) injected into its wells, "with the cathode connected to the well side of the unit and the anode on the opposite side". Bromophenol blue tracking dye was run through the gel at 70 V till it reached the gel's far end. "Gels were stained with ethidium the DNA" (16).

Result & Discussion

This study aimed to investigate the epidemiological distribution of hydatid cyst infections in sheep across various parameters including months, age groups, sex, and sampling locations, in addition to confirming the presence of *Echinococcus granulosus* through molecular diagnosis.

A total of 5547 sheep were examined, and the results revealed significant variation in the distribution of hydatid cysts depending on the month of sampling, age, sex, and anatomical site of infection. The liver and lungs were the most commonly affected organs. Monthly fluctuations in infection rates were observed, with higher incidences reported during the colder month (Table 2,3 ,4 and5) .

Age-wise in sheep between 5–6 years exhibited the highest infection rates, suggesting a possible correlation between age and prolonged exposure. Regarding gender, females showed higher infection rates compared to males. Furthermore, sampling location had an influence on prevalence, with slaughterhouses showing a higher number of infections compared to butcher shops.

Table 2. Monthly Distribution of Hydatid Cyst Infections in Sheep

Month	Total	Site of infection				
Month		Liver	Lung	Kidney	Mesentery	Meat
October	770	401	201	83	20	65
November	746	325	180	86	35	80
December	773	370	204	88	30	81
January	547	235	158	77	26	51
February	690	310	175	98	45	62
March	631	260	175	97	36	63
April	711	370	182	80	11	68
May	679	329	175	85	17	73

Total	5547	2635	1470	664	212	549

Table 3. Distribution of Hydatid Cyst Infections in Sheep According to Age Groups

Age	Total	Site of infection				
		Liver	Lung	Kidney	Mesentery	Meat
1-2 Years	1000	456	243	162	43	113
2-3 Years	1100	501	277	114	40	98
3-4 Years	820	477	261	137	37	104
4-5 Years	1200	565	222	160	32	151
5-6 Years	1427	602	337	188	58	169
Total	5547	2601	1340	761	210	635

Table 4. Distribution of Hydatid Cyst Infections in Sheep According to Sex

Gender	Total	Site of infection				
		Liver	Lung	Kidney	Mesentery	Meat
Male	1100	384	325	187	53	146
Female	4447	2217	1015	574	173	489
Total	5547	2601	1340	761	226	635

Table 5. Distribution of Hydatid Cyst Infections in Sheep According to Sampling Areas

Type	Total	Site of infection				
		Liver	Lung	Kidney	Mesentery	Meat
Butcher	677	421	217	118	35	98
Slaughter house	4870	2376	1023	531	191	537
Total	5547	2797	1240	649	226	635

Molecular diagnosis using PCR confirmed the presence of *Taeniidae gen*. spp. and *E. granulosus* through amplification of specific target genes. As shown in Figure 2 (A,B) bands at 875 bp and 226 bp corresponding to the CO1JP2 and ND1Eg primers, respectively, indicate high specificity and successful amplification, further validating the morphological findings.

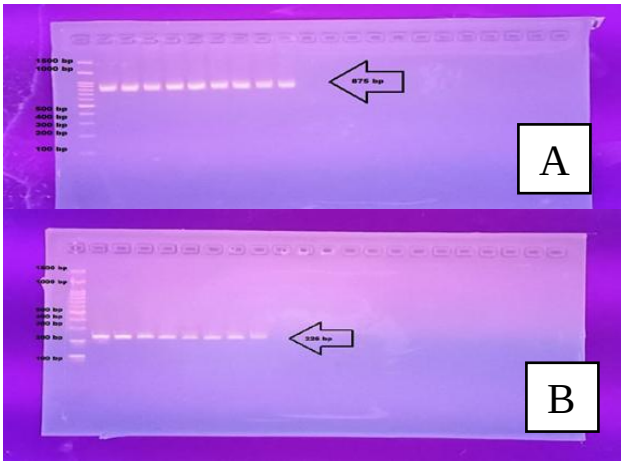

Figure 1. A- A distinct band at 875 bp confirms the presence of *Taeniidae gen*. sp. using the CO1JP2 primer set. B-The clear band at 226 bp indicates successful amplification of *E. granulosus* with the ND1Eg primers. No non-specific bands were observed, indicating high primer specificity. The intensity of the bands suggests efficient amplification of the target DNA regions. Both target fragments were clearly resolved, demonstrating successful PCR and gel electrophoresis conditions. The expected amplicon sizes were successfully obtained, confirming accurate primer design and template specificity. The gel image validates the presence of *Taeniidae gen*. sp. and *E. granulosus* in the tested samples. PCR results were consistent with the anticipated product sizes of 875 bp and 226 bp..

The study conclusions showed that among the sheep samples collected, the G1 genotype of *E. granulosus*, i.e., the strain prevalent in sheep, was the most often detected genotype. Our analysis showed that the *E. granulosus* strain that was most free It does, nevertheless, contradict the information presented in source (17), respectively shows that two distinct strains were found in sheep samples: strain (G1) and buffalo strain (G3), both require cox1 and nad1. Given that AgB component 2 was employed in the present investigation, this discrepancy might be the result of an additional target. often discovered in Al-Diwaniyah matched the findings of an earlier investigation (18). The results of examining and differentiating different strains species *E. granulosus* can be influenced by the techniques employed for its genome sequencing including genomic classification. Variability with the methods used to isolate and grow the parasitic organism, in addition to variability with the genome sequencing methods applied, could lead to discrepancies in the way the particular types are identified. The findings were inconsistent with earlier studies carried out in Iraq, particularly by (19), which found that G1 was present in both humans and animals. The findings showed a hundred percent affinity and full match with the sheep strain (G1).(19)

Since the region is highly prevalent in hydatid cyst (perhaps as a result of external plus livestock-related factors), at Kerbala city the animals especially sheep relevant for researching the AgB2 genome from hydatid cyst . It is possible for

intermediates (sheep) to replace recipients (dogs) to cut off each other and, consequently, the development of the parasite in urban agricultural and pastoral regions where sheep are a major mammal. The infection is also propagated by traditional techniques of animal husbandry with the value domestic slaughter and inappropriate waste removal of diseased parts. the city of Karbala is the perfect place to investigate the genetic and epidemiological biology of *E. granulosus* and to develop targeted diagnostic and control measures because of these discoveries as well as the ideal habitat for parasite growth. AgB polypeptide. AgB is a crucial antigenic target in the host's immune reaction to *E. granulosus*-caused echinococcosis with cysts (CE), which makes this noteworthy. Cystic fluids The agb will component 2 aggregating. Because of the characteristics of CE cystic lesions, AgB component 2 can get released from the parasitic organism and subsequently concentrate in cysts fluids. Due to variations in genetic and physiological features, the use of the antigen B subunit 2 (AgB2) in this study was able to discover genome-wide variations from those found by mitochondrial genes, such as cox1 and nad1. The nuclear genome encodes AgB2, that is directly involved in the control of immunity with is exposed to selection stresses arising through hosting immune systems. This could result in increased variation in genes and host-specific adaptability (11,12). Because of this, AgB2 may prove helpful in identifying extremely modest genotypic variations in host pathogen or antigen connections, as is the case in areas like Al-Diwaniyah where various environment and agricultural conditions are present. As an alternative, the mitochondrial genes such as the amount of cox as well as nad1 are highly conserved and mostly utilized for massive amounts genealogical classes of *Echinococcus granulosus* genotype (e.g., G1-G10) (20). Although host- particular modifications or functional distinctions that might be useful to assess local epidemiological as well as immunological activity may be missed by these markers, which means they may prove helpful for international strain identification. By comprehending immunological and pathogenic dynamics, the emphasis on AgB2 seeks to enhance diagnostic and control techniques in addition to characterizing the genetic diversity of E. *granulosus* locally (21).

AgB component 2 may combine form a shape which becomes more immunogenic , capable of eliciting a more potent immunological response—than the particular monomers version thanks for this aggregation. Both (22) and (23) discovered that the G1 genotypes of Echinococcus granulosus was the most common in northeastern Iran. Throughout the organism's lifetime, Hejazi et al. concentrated on isolation of primary hosts (dogs) as well as sheep and dogs. For quick and accurate sheep genotyping, and Mohaghegh et al. employed cox1-based qPCR HRM, concentrating on mitochondrial genes because they are conserved and chronologically classifiable. The host's immune reaction against the pathogen may be influenced by the aggregating version of Agb, which stands component 2, which may be a more powerful immunological antigen compared to the monomers version. It is currently shown that transgenic AgB has therapeutic capability.

These findings suggest that recombinant AgB should be recommended for the serological diagnosis of cystic echinococcosis. In summary, the key points highlight the genetic similarities between local and international parasite isolates, the expression and potential immunogenic properties of the AgB antigen, and the diagnostic utility of recombinant AgB, all of which are important considerations in understanding and managing cystic echinococcosis(24). The similarity between the E. *granulosus* strains under study and global strains may be attributed to the fact that the aforementioned gene does not show any difference in its nucleotide sequence (25).

A distinct band at 875 bp confirms the presence of Taeniidae gen. sp. using the CO1JP2 primer set, consistent with findings reported by Nakao *et al*. (26) (27), who designed the primers to target the mitochondrial cox1 gene in various Taeniidae species. Similarly, the amplification of a 226 bp fragment using the ND1Eg primer set confirms the presence of Echinococcus granulosus, aligning with the results of Dinkel *et al.(*28) , who demonstrated the effectiveness of nad1-based primers in specifically identifying E. granulosus. The absence of non-specific amplification supports the high specificity of both primer sets, as previously validated in comparative molecular studies targeting cestode mitochondrial genes. The strong intensity and clarity of the bands indicate efficient DNA amplification and optimal PCR conditions, corroborating the protocols established in earlier diagnostic approaches for cestode detection (29). These results not only validate the utility of the CO1JP2 and ND1Eg markers for accurate species identification but also reinforce their diagnostic reliability as demonstrated in prior epidemiological surveys and genetic characterization studies .

CONCLUSION

The molecular characterization of Echinococcus granulosus isolates from sheep in Holy Kerbala City has provided valuable insights into the genetic diversity and local epidemiology of the parasite. Using mitochondrial gene sequencing (e.g., CO1 or ND1), the isolates were identified and compared with global reference strains, confirming the presence of specific genotypes prevalent in the region, most notably the G1 (sheep strain). These findings highlight the continued circulation of zoonotic strains within the livestock population and underscore the importance of implementing targeted control strategies, including improved slaughterhouse hygiene, public awareness, and coordinated veterinary-public health measures. Further molecular surveillance is recommended to monitor genetic variations over time and assess potential risks to human health

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