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SDS-protein and 18S rRNA gene, and phylogeny-based identification of hydatid cysts in human and animal samples

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Abstract

Hydatid disease is a significant and widespread infectious disease prevalent in many countries where there is inadequate control of the adult tapeworm; *Echinococcus* spp, in dogs, which are the definite hosts. The disease is caused by the presence of hydatid cysts in intermediate hosts, such as humans and some farm animals. In Iraq, infected dogs are highly likely to transmit the infection to humans and some farm animals. The study aimed to identify the presence of hydatid cysts in human and animal samples. The research involved collecting thirty hydatid cysts from human livers and ovaries, as well as from animal livers and lungs. The samples were subjected to SDS-based protein detection, PCR, and gene sequencing of the 18S rRNA gene. The SDS-Coomassie-blue findings demonstrated a 100% (15/15) positive identification of the cystic protein of all specimens. The PCR results confirmed the identity of the parasite with 100% accuracy. Sequencing and phylogenetic analysis of 10 PCR products from both human and animal samples revealed close nucleotide identity to global isolates previously deposited in GeneBank. These findings indicate a high occurrence of hydatid cysts in the examined humans and animals, which have evolved or exhibit close genetic similarity to global strains.

Keywords

Echinococcus; hydatid disease; multilocularis

Introduction

Hydatidosis, caused by *Echinococcus granulosus* and *E. multilocularis*, is a parasitic disease transmitted from carnivores to humans. The pathogens are the larval stages of tapeworms from the genus *Echinococcus*, primarily members of the family Taeniidae. A hydatid cyst results from the larval stage (oncosphere) of the *Echinococcus* tapeworm. The hydatid cyst is a fluid-filled cyst caused by the growth of the tapeworm oncosphere in the affected organ or tissue (1). The larval stage develops in a wide range of intermediate hosts, mainly humans, while the adult form is typically found in carnivorous animals (2).

Echinococcosis (Hydatidosis) is widely distributed in regions where sheep and cattle are raised around the globe. *E. granulosus* is prevalent worldwide, with the highest prevalence found in three regions of Eurasia: Africa, Australia and South America. *E. multilocularis* is distributed in several regions of the Northern hemisphere (1), occuring endemically in central Europe, most of northern and central Eurasia, parts of North America and Northern Africa (3). A study demonstrates that the prevalence of hydatidosis

was 46% in medium-altitude areas, 24% in highland areas and 25% in lowland areas. Similarly, another study found that 14.45% of stray dogs were carrying *E. granulous*. These findings suggest that hydatidosis is highly prevalent in Africa, which is consistent with numerous previous studies (4). As a global parasitic disease affecting humans, echinococcosis (hydatidosis) poses significant public health problems. The most epidemiologically prevalent form is caused by *E. granulosus*, while the most aggressive form is caused by *E. multilocularis*. Alarming increases in health risks from echinococcosis have been reported from several regions worldwide, indicating a growing public health concern (5).

Beyond its implications for the health of animals and humans, hydatidosis causes economic losses due to the condemnation of organs and carcasses, and the cost of controlling and treating the disease in both animals and humans (6). Veterinary medicine plays a crucial role in studying hydatidosis and controlling its spread, as the disease can be managed by regulating the parasite in animals. The *E. granulosus* lifecycle involves various definitive and intermediate hosts. Definitive hosts include a wide range of canids, such as dogs, wolves and jackals, but potentially any carnivore could be involved. Intermediate hosts vary widely and include sheep, goats, cattle and pigs. The definitive host becomes infected with the larval stage of the parasite, which, after developing, is shed in the feces. The new larvae or embryos are then ingested by intermediate hosts. Definitive hosts can also be infected by ingesting the embryos through external parasites or by consuming the larvae in the organs of intermediate hosts (1). Cooperative efforts among veterinarians and public health workers are vital for controlling the disease (7). Due to the absence of signs and symptoms (subclinical) in both definitive and intermediate hosts, hydatidosis is difficult to diagnose, increasing the possibility of transmission. To control any infectious agent, it is essential to understand the transmission cycle that maintains the agent in the natural environment (8-10).

Hydatid disease is a significant and widespread infectious disease in many countries where there is inadequate control of the adult tapeworm; *Echinococcus* spp, in dogs, which are the definitive hosts. The disease is caused by the presence of hydatid cyst in intermediate hosts, such as humans and some farm animals. In Iraq, infected dogs are highly likely to transmit the infection to humans and animals. This study was conducted to identify the presence of these cysts in human and animal samples.

Materials and Methods

Samples and DNA extraction

Thirty hydatid cyst specimens were collected directly from the livers and ovaries of human patients at the Hospital of Al-Muthanna after surgical removal of the cysts and from the livers and lungs of sheep at an abattoir. These patients exhibited clinical symptoms including right abdominal pain, fever, nausea, vomiting, chronic cough, dyspnea and pleuritic chest pain. The samples were sent to the laboratory using aseptic instruments and containers for comprehensive analysis. First, DNA extraction was performed on each sample according to the manufacturer's instructions for the Qiagen kit. The DNA samples were then analyzed using a NanoDrop spectrophotometer and stored in a -20 °C freezer until used for PCR.

SDS

Sodium dodecyl sulfate polyacrylamide gel electrophoresis with Coomassie Blue staining is one of the most commonly used methods for the separation and visualization of proteins by their molecular weight. The step-by-step protocol includes the preparation of gel solutions, casting of the gel and sample preparation. Preparation of the gel solution for separation involves making solutions for the stacking gel and resolving gel. The resolving gel is prepared with a 10-15% acrylamide concentration and poured into the casting frame. A well is placed on the unpolymerized gel, which is then overlaid with isopropanol using wafer blocker. Once the gel has polymerized, the stacking solution, with a 4-5% acrylamide concentration, is added.

The protein sample is prepared by mixing the protein sample with SDS sample buffer. This mixture is then heated to boiling to denature the proteins. Once the gel has polymerized, the sample contents and molecular weight markers are loaded into the wells on the gel. Electrophoresis is then carried out using an electrophoresis setup to separate the components by their molecular weight.

After the electrophoresis runs, the gel is stained by adding a dye such as Coomassie blue and left overnight to allow the dye to bind to the proteins. Excess stain is removed by washing the gel with a destaining solution, which enhances the contrast and facilitates visualization of protein bands.

PCR

A further test using Gradient PCR detected evolution in *Echinococcosis* spp. All PCR products underwent confirmation. The PCR primers targeted the cox1 gene in mitochondrial genomes to detect *Echinococcus* spp., using the following primers: Forward (F) GTTTAGGGGCTGGTGTTGGT and Reverse (R) ACCCTGCTACCCAAACACAC, amplifying a 499 bp DNA fragment (PP511909.1). The PCR program was set up with an initial denaturation at 95°C for 5 min, followed by 35 cycles: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds. A final extension step was performed at 72°C for 7 min. The PCR was conducted using a Thermo cycler (Fisher, Germany). Subsequently, the PCR product were loaded onto a 1.5 % agarose gel and electrophoresed for 60 min at 80 Volts. Interpretation was carried out using a UV trans illuminator to visualise the DNA bands.

Cox 1-Gene sequencing

A 40 µl sample of purified Cox1 PCR product was prepared for DNA sequencing with assistance from Macrogen Company in Korea. The unedited sequences were analysed

using the NCBI BLASTn engine to determine the level of homology between the target sequence and Contig sequence of Cox1 gene of *Echinococcosis* spp genome sequences. Finally, MEGA X software (version: MEGA, 11.0.11) was utilized for multiple alignments to construct the phylogenetic tree and compare genetic divergences between the studied isolate and global strains of *Echinococcosis* spp.

Results

The SDS- Coomassie-blue findings confirmed the presence of cyst protein in 15/15 (100%) human and animal specimens. The PCR results also verified the identity with 100% accuracy (Fig. 1).

The available Cox1 gene sequences of *Echinococcus* isolated in this study will be directly compared with sequences deposited in GenBank. This comparison will focus on similarities and variabilities, shedding light on how the new isolates relate to previously published and sequenced strains, and their geographical and host distribution. Furthermore, the Cox1 gene sequences and their genotypes from multiple specimens will be phylogenetically analyzed by constructing phylogenetic trees using methods such as maximum likelihood. The resulting trees will visualize the evolutionary relationships among the Cox1 gene sequences. Strains with close similarities in the Cox1 sequence will indicate a close evolutionary relationship and may suggest clusters among the isolates. These clusters warrant further investigation to determine whether they represent distinct groups from specific geographic regions or new lineage. Sequencing of 10 PCR products from each human and animal revealed close similarity to global isolates previously deposited in GeneBank, particularly from Italy (Fig 2, 3).

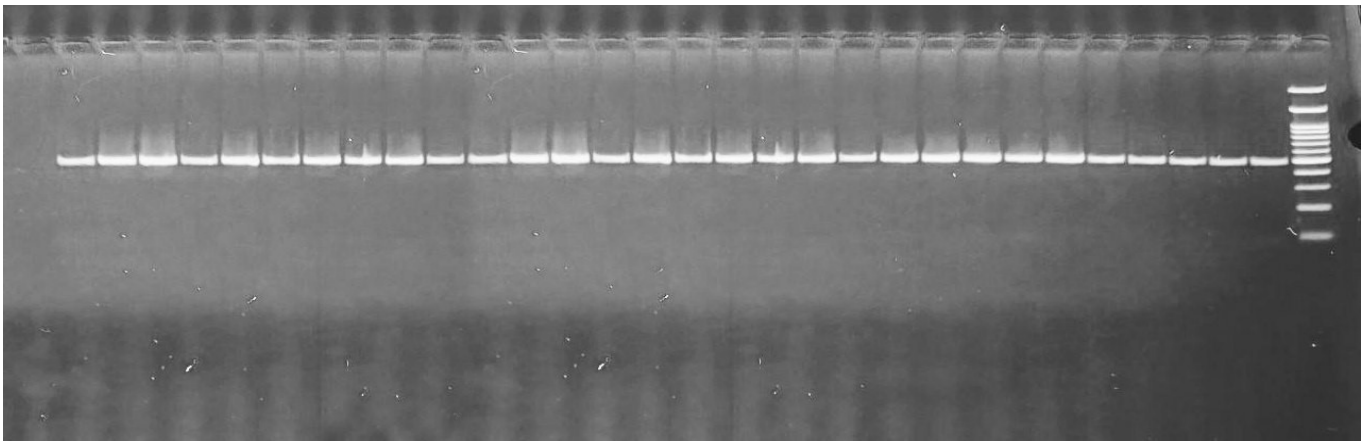

Fig. 1. Image of agarose gel electrophoresis of *Echinococcus* spp isolated from humans and animals. Bands represent positive samples at 499 bp of Cox1 gene. Ladder: 1500 bp.

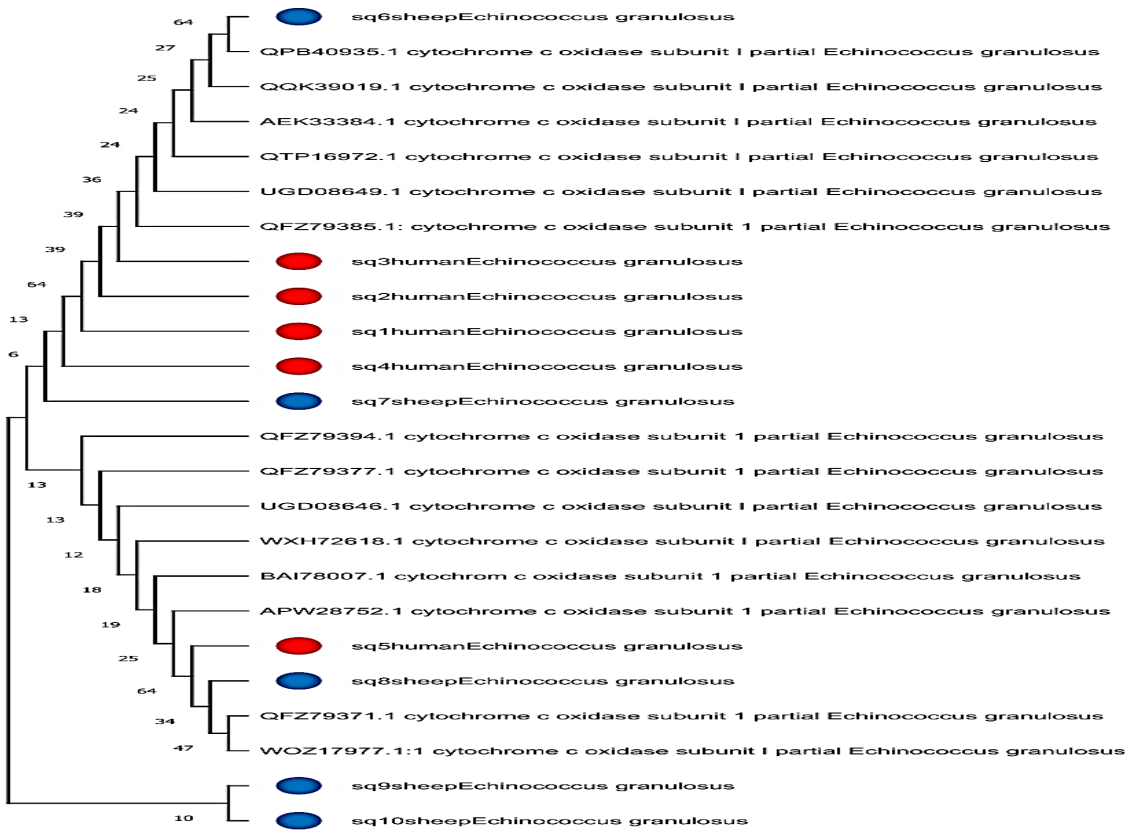

Fig. 2. Phylogenetic tree of *Cox1* gene of *Echinococcus* spp isolated from humans and animals. Color filled circles represent the current study isolates.

Fig. 3. Alignments of *Cox1* gene of *Echinococcus* spp isolated from humans and animals with world isolates.

Discussion

The larval form of the tapeworm, *Echinococcus granulosus*, affects both humans and animals, causing hydatid cysts. Accurate identification of these cysts is crucial for diagnosis and treatment. In the laboratory, SDS-Coomassie Blue staining is used to visualize proteins in biological specimens. This technique involves Sodium Dodecyl Sulfate (SDS) to denature proteins, unfolding their structure. Coomassie Blue then binds to the unfolded proteins, resulting in a blue coloration. This staining method has shown promising results for identifying hydatid cysts, allowing visualization of the distinct laminated layer and protoscolices, which are crucial for cyst identification and classification. The PCR or polymerase chain reaction is a molecular technique used to amplify (replicate) a targeted DNA sequence. Specifically, targeting the 18S rRNA gene can aid in identifying hydatid cysts. In the current study, SDS and PCR achieved a 100% identification rate for hydatid cysts.

Increased numbers of studies conducted in developing countries in the Middle East have also revealed a high prevalence of this disease. For instance, studies on the Northern provinces of Iran in 2005, estimated the prevalence to be 2.06%, 2.36%, and 2.82% respectively (11 -13). Hydatidosis results in significant economic and health burdens worldwide due to the costs incurred for patients care and treatment. The different strains of the *Echinococcus* genus exhibit highly genetic polymorphic, as recent molecular studies have shown significant diversity in genotypic profiles of the parasite. Genotypic diversity influences various parameters such as the life cycle, transmission dynamics, biochemical characteristics, pathogenesis and susceptibility to specific drugs (14, 15). Therefore, it is essential to determine the prevalence and genotype of echinococcosis in each geographical region. These findings can inform the development of health strategies, preventive measures, and the creation of vaccines with improved sensitivity and specificity for diagnosing and treating specific genotypes (16).

Upon reviewing the findings, 2 strains, G1 and G6, were identified in various intermediate hosts including sheep, cattle, goats, camels and buffalo (17). G1 was found to be the predominant strain across all intermediate hosts. Another study aimed to determine the dominant strain of

E. granulosus in West Azerbaijan, Iran. Through sequence analysis, they reported that all cysts from sheep, goats and cattle belonged to G1, while buffalo cysts were identified as G3 in this region (18). In a study, 49 sheep, 28 cattle, and nine human specimens from Zanjan province, Iran, were examined and G1 was identified as the most prevalent genotype (19). This conclusion was drawn from PCR-RFLP analysis of a 433 base-pair ITS1 fragment.

It was investigated that the prevalence of metacestodes in various intermediate hosts in the eastern Punjab province of Pakistan using the mitochondrial cox1 gene (20). Recently, through southern blotting and molecular fingerprinting, it was determined that out of 10 dog parasites, G1 was the predominant genotype in 2 regions of Kenya (21). The high prevalence of intestinal disease in the Middle East was revealed by a study conducted in the northern region of Iraq, neighboring Iran (22). They found that the G1 genotype was predominantly prevalent in sheep, while G3 was dominant in buffalo.

The phylogenetic tree illustrates the evolutionary relationships among different sequences of the cytochrome c oxidase subunit I gene from *Echinococcus granulosus* isolated from humans and sheep (16). Branch lengths on the tree indicate evolutionary distances among clades, with longer distances indicating greater genetic divergence. Bootstrap values are depicted in red numbers next to branches, with higher numbers indicating stronger support. Additionally, all sequences marked in red dots represent sequences from human hosts. Clusters at the terminal end with a bootstrap value of 64 indicate very close genetic relationships among or between sequences from human hosts. Furthermore, sequences from sheep livers, marked in blue, form distinct clusters with a highly supported bootstrap value of 99. This suggests that *Echinococcus granulosus* infecting different hosts exhibit genetic differences and the observed phylogenetic structure is strongly supported (22).

Conclusion

This study underscores that hydatid disease poses a serious public health challenge in Iraq, primarily due to ineffective control of the life cycle of *Echinococcus* spp. in dogs, the definitive hosts. Twenty-four human and six animal cysts were examined for the presence of hydatid cysts. Through SDS-based protein detection, PCR, and gene sequencing of the parasite's 18S rRNA gene, the study confirms a high prevalence of this disease. SDS-Coomassie Blue staining successfully identified cystic proteins of the parasite with 100% accuracy, while PCR results unequivocally confirmed the identity of the parasite with 100% certainty. Phylogenetic analyses revealed that Iraqi isolates are closely genetically related to global strains. These findings confirm that the occurrence of hydatid cysts in Iraq shares close genetic relatedness with strains found globally.

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Not applicable

Authors' contributions

All authors participated in all parts of the current study.

Compliance with ethical standards

Conflict of interest: No competing of interest is present in the current study.

Ethical issues: All procedures were followed as they are approved by the Ethical Committee in the College of Veterinary Medicine, Al-Qassim Green University, Babylon Province, Iraq.

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