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Molecular Identification and Genetic Characterization of Hydatidosis in Egyptian Cases

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ABSTRACT

Background: Hydatid disease, caused by the larval stage of *Echinococcus granulosus*, is a globally significant zoonosis with notable public health and economic impacts. Despite its prevalence in Egypt, research on the genetic diversity of *Echinococcus* in humans is limited.

Objectives: This study aims to achieve molecular identification and genetic characterization of hydatidosis in Egyptian cases through PCR analysis, followed by DNA sequencing and phylogenetic assessment.

Patients and methods: A descriptive observational cross-sectional study was conducted on 40 hydatid cyst samples from Egyptian patients. These samples were obtained from liver cysts via percutaneous aspiration using the PAIR technique under ultrasonographic guidance, along with three surgically excised hydatid cysts. The aspirated fluid was examined microscopically. Indirect hemagglutination test (IHAT) was done as a routine preoperative test for the suspected cases of the disease. DNA from the protoscolices was used in a PCR targeting cytochrome oxidase subunit 1 (COX1) followed by sequencing and phylogenetic analysis that successfully submitted in NCBI GenBank.

Results: IHAT results revealed that only 27.5% of patients tested positive, while 72.5% were negative. PCR analysis of DNA extracted from the human cyst samples demonstrated a 90% positivity rate (36/40) for COX1 gene. Subsequent sequencing and phylogenetic analysis confirmed a 100% homology with *E. granulosus* genotype G1 previously obtained from sheep in Egypt.

Conclusions: Zoonotic potential of G1 sheep strain of *E.granulosus* as a predominant genotype infecting the humans in Egypt. The reported data could be used for proper diagnosis and control of hydatidosis in Egypt.

Keywords: Hydatid cyst, *Echinococcus granulosus*, Molecular, COX1, Zoonotic, Egypt.

INTRODUCTION

Hydatid disease, also known as hydatidosis or echinococcosis, is attributable to the larval stage of *Echinococcus granulosus* [1]. This infection demonstrates widespread prevalence across diverse regions worldwide. Echinococcosis was classified by the World Health Organization (WHO) among the 17 neglected tropical diseases prioritized for either elimination or stringent control measures [2]. The inclusion of cystic echinococcosis (CE) within the WHO's comprehensive framework for addressing neglected diseases underscores its profound implications for public health and socioeconomic stability [3].

The life cycle of the parasite involves two distinct mammalian hosts. The larval form, known as the hydatid cyst, develops within the internal organs of various mammalian species, including humans, who become infected through the inadvertent ingestion of eggs. In contrast, the adult stage of the parasite resides within the small intestines of carnivores, predominantly dogs, which serve as definitive hosts [4].

Hydatid cysts exhibit a marked predilection for the liver, followed closely by pulmonary involvement, though they possess the capability to affect an extensive array of organs within the human body. The clinical presentation spans a broad continuum, ranging from an entirely asymptomatic state to conditions of profound severity or even mortality [5].

The diagnosis of hydatid cyst is based on several items criteria, including history of residence in endemic areas, clinical examination**,** imaging techniques, histopathology, serology and nucleic acid detection [6] such as immunoelectrophoresis, double diffusion in agar, indirect hemagglutination, enzyme-linked immune sorbent assay (ELISA), immunoblotting, and direct immunofluorescence test [7]**.**

The problem is that antibodies remain for years in patient's sera and it is not easily to differentiate between the acute and chronic phase of the disease or to follow up the patient after treatment or surgery [8]**.** Some patients with cystic echinococcosis do not demonstrate a detectable immune response [9]**.**

The polymerase chain reaction has the advantages of being accurate, sensitive and requiring only DNA from viable or non- viable organisms for positive results [10].

Understanding transmission dynamics of the parasite between intermediate and definitive hosts relies heavily on the genetic profiling of *E. granulosus* populations, a process that also plays a pivotal role in enhancing the diagnosis and management strategies for CE [11]. Within the realm of genotyping methodologies,

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sequencing has emerged as the definitive benchmark [12], demonstrating remarkable efficacy in the molecular identification and characterization of taeniid tapeworms through the analysis of mitochondrial DNA (mtDNA), specifically targeting COX1 loci [13].

Ten distinct genotypes: *E. granulosus* (sensu stricto), encompassing the sheep strain (G1–G3); *Echinococcus equinus*, representing the horse strain (G4); *Echinococcus ortleppi*, associated with the cattle strain (G5); *Echinococcus canadensis*, which includes the camel strain (G6), pig strain (G7), and cervid strains (G8 and G10); and *Echinococcus felidis*, linked to the lion strain, which has no genotype [11] were designed based on phenotypic characteristics and molecular analyses.

Variations among *Echinococcus* species exert profound effects on numerous aspects of the parasite's biology and epidemiology, including its life cycle, growth kinetics, host specificity, pathogenic mechanisms, transmission patterns, drug susceptibility, antigenic properties, epidemiological behaviour, and approaches to control and prevention. Recognizing the predominant strain or strains within a specific region is crucial for implementing effective control and eradication measures, as well as for advancing the development of diagnostic tools, vaccines, and therapeutic strategies. However, a significant gap persists in both regional and global epidemiological and molecular research aimed at determining the prevalence and genetic diversity of *Echinococcus* species [14].

The present study aimed to achieve molecular identification and genetic characterization of hydatidosis in Egyptian cases through PCR analysis, followed by DNA sequencing and phylogenetic assessment.

MATERIALS AND METHODS

This descriptive, observational, cross-sectional study was conducted on human hydatid cyst samples collected over a one-year period, spanning from January 2023 to December 2023.

Inclusion criteria: The study adhered to WHO guidelines and encompassed solitary liver cysts (>5 cm) identified via ultrasound, large liver cysts (>5 cm) with multiple daughter cysts, superficially located cysts with a high risk of spontaneous or trauma-induced rupture, cysts with biliary tree communication, viable cysts demonstrating evidence of active infection, complex cysts and cysts exerting local pressure on adjacent organs [15].

Exclusion criteria: Patients with inaccessible liver cysts, pregnant women, individuals with liver cysts smaller than 30 mm in diameter, and those with active malignant disease [15].

Sampling collection: The study included forty patients diagnosed with cystic echinococcosis who were attending the Interventional Radiology Unit of the Tropical Medicine and Surgery Departments at Kasr El-Aini Hospital, Cairo University, as well as the Hepato-Gastroenterology and Infectious Diseases Department at Al-Zahraa University Hospital, Al-Azhar University, as well as Theodore Bilharz Research Institute (TBRI) Hospital at Giza, Egypt. Cystic fluid samples were collected under complete aseptic conditions by percutaneous aspiration (PAIR technique) guided by ultrasonography from liver cysts and 3 samples of a surgically removed hydatid cyst. IHA test was done as a routine preoperative test for the suspected cases of the disease.

Direct parasitological study: Cyst fertility was assessed by microscopic examination of the aspirated cystic fluid for detection of the presence of protoscolices according to **Zhang *et al.*** [16] where the fluid was transferred into a sterile suitable (15 or 50 ml) labelled tubes, then examined by direct wet mount microscopic examination of the deposit after centrifugation through 10x and 40x magnification.

Supernatants were discarded and the rest of each sample (originated from a single cyst) was transferred into a sterile test tube and stored at -20°C for further molecular examination.

Molecular techniques:

1. DNA extraction was done using the DNeasy Blood and Tissue Kit (Qiagen, Germany) following the kit instructions. The purified DNA was eluted in 50 µL of the elution buffer included in the kit.

2. Polymerase Chain Reaction (PCR) amplification and Gel Electrophoresis: The mitochondrial COX1 gene, was amplified in a 25µl reaction mixture containing 12.5 µl of 2×*EasyTaq*® PCR SuperMix (Cat. AS111-01/11, Trans Co., China), 0.5 µL (10 µM) of each primer (**Table 1**), and 2 µL of target DNA with PCR conditions as showed in **(Table 2)**. The In Genius3 gel documentation system (Syngene, UK) was utilized to analyse the PCR products. Separation of them was carried out on a 1% agarose gel, followed by staining with ethidium bromide. A 100 bp molecular weight ladder was used as a reference for comparison.

Table (1): PCR primers and probes utilized for gene amplification in the study

Gene	Sequence (5´-3´)	Amplicon size (bp)	Reference
COX1	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	**450**	**Bowles *et al.*** [17]

Table (2): PCR cycling parameters for gene detection in the current study

Gene	Initial Denaturation	Denaturation	Annealing	Extention	Final Extention	Cycles
COX1	95˚C 2min	95˚C 20sec	55˚C 30sec	72˚C 45sec	72˚C 10min	40

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3. DNA sequencing and phylogenetic tree construction

Two amplified PCR products corresponding to the COX1 gene were purified using the Gene JET™ Gel Extraction Kit (K0691, Thermo Fisher, USA) and subjected to sequencing on 3730_L capillary sequencers (Applied Biosystem, USA) through Macrogen Company (Korea). The sequencing process employed a bidirectional approach, leveraging both forward and reverse primers from the initial PCR to ensure accuracy. The nucleotide sequences obtained were analyzed with BioEdit 7.0.4.1 and ClustalW2 software (http://www.clustal.org/). Alignment of these sequences with reference Echinococcus spp. genes was performed, and a neighbor-joining analysis of the aligned sequences was carried out using the CLC Sequence Viewer 6 program.

Ethical consideration

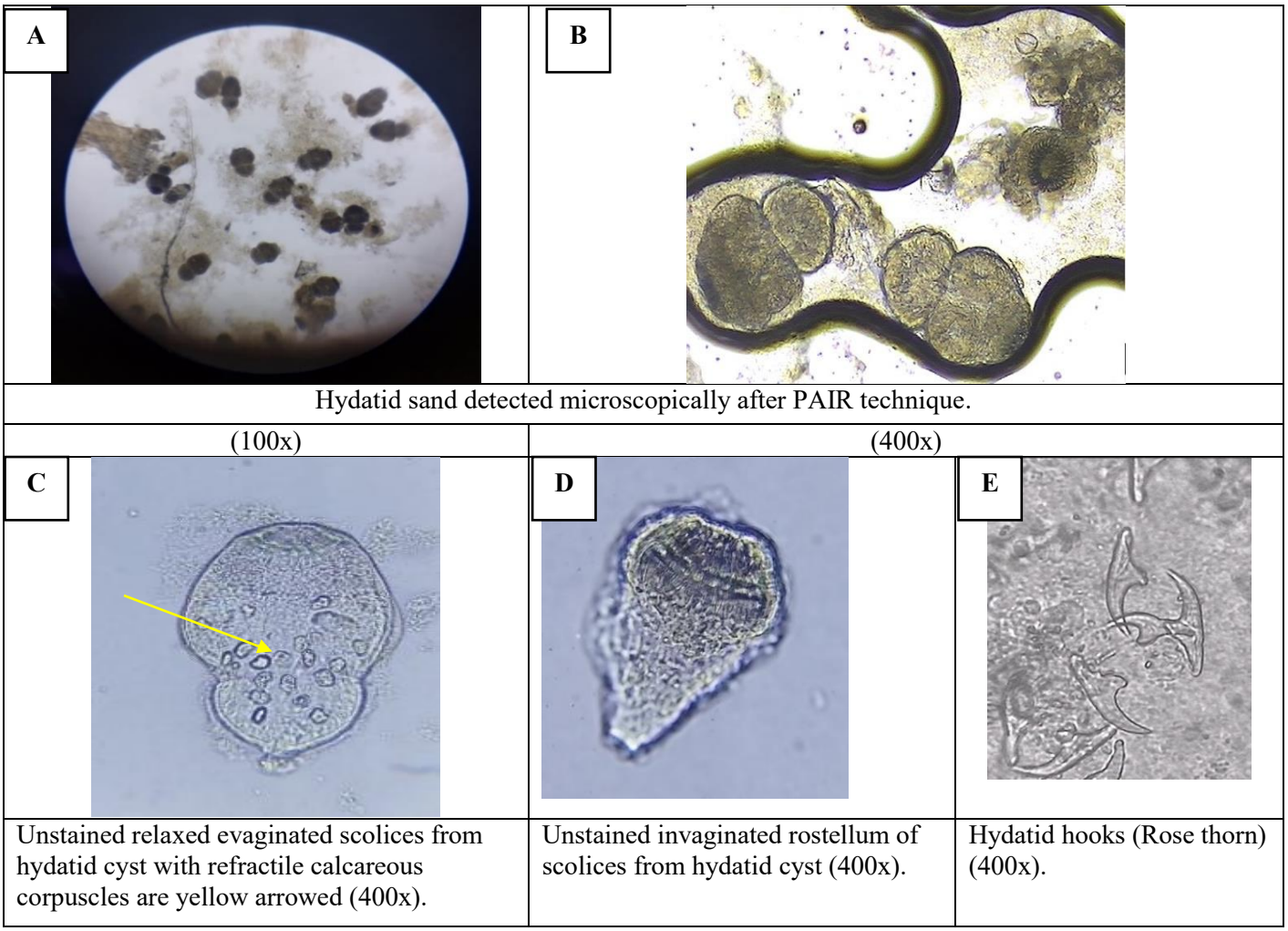
The study was conducted in full compliance with the ethical principles outlined in the Declaration of Helsinki and relevant guidelines for good clinical practice. It adhered to the directives and regulatory framework established by the Egyptian Ministry of Higher Education. Under IRP No. 1801, the protocol was thoroughly evaluated and approved by the Ethics Committee of the Faculty of Medicine for Girls, Al-Azhar University. Following a thorough and clear explanation of the study objectives, accompanied by addressing any questions or concerns, each participant provided informed written consent. PAIR or surgery in conjunction with albendazole chemotherapy were previously employed to diagnose and treat patients.

Statistical analysis

Data were meticulously gathered, thoroughly reviewed, systematically coded, and analyzed utilizing IBM's Statistical Package for the Social Sciences (SPSS), Version 27. For quantitative variables, parametric data were expressed as means, standard deviations, and ranges, whereas non-parametric data were summarized using medians and interquartile ranges (IQR). Qualitative variables were presented as frequencies and percentages.

RESULTS

Microscopic evaluation of hydatid cystic fluid demonstrated that 38 out of 40 samples (95%) tested were positive. The remaining two samples (5%), were initially negative under microscopic analysis, and later they were confirmed to be positive following examination of the cyst wall, which revealed the presence of both germinal and laminated layers.



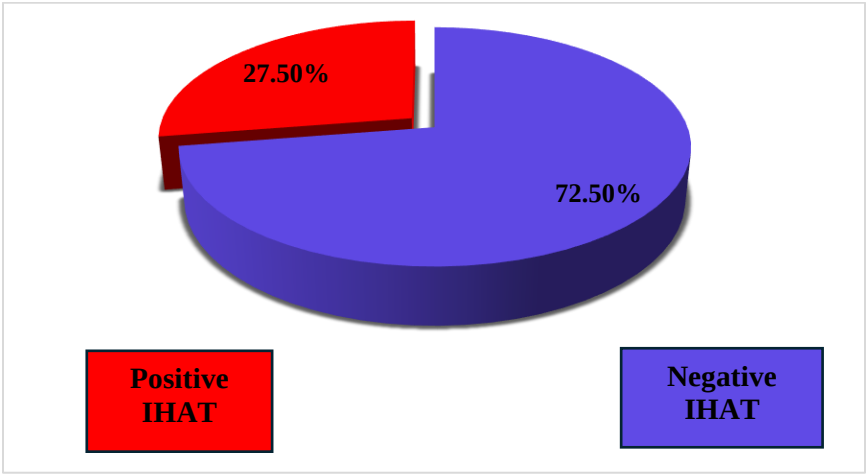


Figure (2): Percent of positivity of IHA test in studied cases.

This figure shows the percentage of positivity to hydatid antibodies which was done as a routine preoperative test for the suspected cases. The data collected showed that the investigated cases were positive only in 11 cases (27.5%) while 29 cases were negative (72.5%).

Using PCR on DNA samples, a fragment of about 450 was amplified targeting *COX1*.

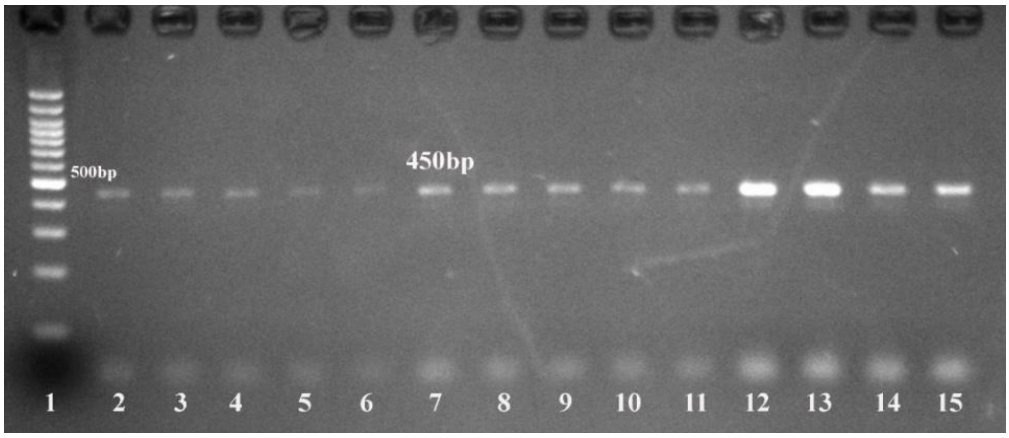


Figure (3): Agarose gel electrophoresis with 100-bp DNA ladder. PCR analysis of the *COX1* gene revealed a 450 bp band. Lane 1: 100 bp ladder, Lanes 2 -15: positive samples.

The partial sequences of the Cox1 gene from our two isolates were submitted to GenBank with accession numbers PP728351 and PP728352. These sequences exhibited 100% homology with the *E. granulosus* genotype G1 identified in sheep from Egypt (AB921090).

DISCUSSION

Hydatid disease, an endemic and often overlooked zoonotic condition, is likely reported across various regions of Egypt. Molecular characterization of parasites retrieved from various intermediate hosts, including humans, is crucial to addressing this knowledge gap. Thus, a thorough and extensive investigation is warranted. Such analysis is indispensable for a detailed understanding of their role in the epidemiology of CE within the region [3].

In this study, microscopic analysis identified scolices or hooks in 38 out of 40 (95 %) of samples. The remaining 5% (2 samples) initially yielded negative results through microscopic examination but were later confirmed positive following evaluation of the germinal layers.

The findings of a study by [18] align with our results, as they observed the presence of hooks in 18 out of 19 human samples examined microscopically. Notably, one sample that initially tested negative was subsequently confirmed positive following an evaluation of the germinal layer. Also, some authors [19] studied 40 human samples and found that all isolates were fertile.

To successfully establish itself within the host, the parasite's germinal layer generates the laminated layer and promotes protoscoleces formation, ensuring the continuation of its life cycle. However, certain cysts fail to generate protoscoleces and are classified as infertile cysts [20].

In our study, 5% of samples were initially negative upon microscopic examination but were later confirmed positive following germinal layer analysis. These findings harmonize with those reported by **Beigh *et al.*** [21] who reported that histopathological examination constitutes a golden tool for diagnosis of hydatidosis.

In the current study, the percentage of positive serology to hydatid disease using IHA test was positive only in 27.5% while 72.5% were negative. These results align with the findings of **Toraman *et al*.** [22], who investigated anti-*E.granulosus* antibodies in serum samples from suspected patients using the IHA method. Their study reported a seropositivity rate of 12.9% among the patients.

According to **Mihmanli *et al.*** [23] IHA is typically non-specific and is beneficial when used in conjunction with other investigations.

Variations in seropositivity and seronegativity among different patient groups can be attributed to the intricate antigenic composition of hydatid cysts [24]. Additionally, the formation of immune complexes may contribute to reduced seropositivity when using the IHAT [25].

The accurate detection of infection requires the implementation of DNA-based molecular assays that combine high sensitivity and specificity with cost-effectiveness. Advances in molecular and biochemical methodologies have enabled the development of diverse techniques for the precise identification and characterization of *Echinococcus* strains [26].

Using PCR on cyst DNA samples, a fragment of about 450, bp were amplified targeting *COX1* where 36/40 (90%) were positive to *COX1*, gene. The findings of this study are in accordance with those reported in previous research by **Ismail *et al.*** [27] Who found that that PCR amplification of the COX1 revealed band measuring 450 bp.

Mirahmadi *et al.* [28] supported these findings, reporting successful amplification of the COX1 gene in 48 out of 55 samples. Also, **Koohestan *et al.*** [29] reported that COX1 gene was successfully amplified in 16 (45.71%) DNA samples from paraffin-embedded human tissue samples.

Genetic characterization of *E. granulosus* populations plays a crucial role in enhancing the diagnosis and management of cystic echinococcosis [11].

Moreover, the mitochondrial COX1 gene is considered a highly suitable marker for assessing genetic diversity and conducting haplotype analysis. Within the same species, its evolutionary rate is sufficiently slow to maintain stability, yet adequately rapid to enable clear differentiation among distinct species. As a result, the mitochondrial COX1 gene has been selected as a key tool in our research for distinguishing between helminth species and creating reliable DNA identifiers [30].

In the current investigation focusing on the COX1 gene, two human isolates (PP728351 and PP728352) demonstrated 100% homology with *Echinococcus granulosus* genotype G1, previously identified in sheep from Egypt (AB921090). These findings are supported by many global studies that identified the G1 genotype as the most common genotype causing human CE to humans [31].

Several studies conducted in various regions of the Middle East, including Iran, have frequently identified *E. granulosus* sensu stricto (*E. granulosus* s. s.) G1 as the most prevalent strain. [32]. Furthermore, the sequencing analysis performed by **Koohestan *et al.*** [29] confirmed that all examined samples belonged to the *E.granulosus* sensu stricto complex, specifically genotypes G1 and G3.

In a similar vein, **Yousefi *et al.*** [33] investigated human CE in Southwest Iran and revealed that the G1 genotype was the exclusive genotype detected across all analyzed samples. The phylogenetic analysis of ***Hamamcı et al.*** [34] based on *COX1* gene region revealed *E. granulosus s. s.* (G1-G3) complex.

Similarly, **Khalifa *et al.*** [35] reported detection of G1 genotype of human cystic echinococcosis in Egypt. According to **Abdelbaset *et al***., the Egyptian population has been found to harbour only three genotypes: G1, G6, and G7 [3].The extensive range of intermediate hosts is likely a key factor contributing to the global predominance of the G1 genotype, as it facilitates broader circulation within the environment [11, 31].

These findings suggest that the G1 genotypes of *E. granulosus* sensu stricto are the most dominant in Egypt, with their life cycle being shaped by interactions involving both wild and domestic animals.

CONCLUSION

Zoonotic potential of G1 (sheep strain) genotypes of *E.granulosus* as a predominant genotypes infecting the humans in Egypt. From a One Health perspective, these data may have considerable implications for shaping control strategies for human hydatidosis, as well as guiding future molecular and biological research.

Conflict of interest: None.

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