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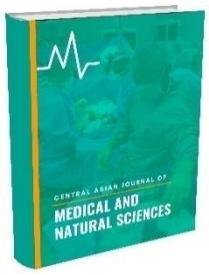

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Phylogenetic Tree Analysis Based on Partial Gene Sequencing of the Mitochondrial Cytochrome C Oxidase Subunit I (*COX1*) Gene of Hydatid Cysts from Patients, Wasit Province, Iraq

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Abstract: **Background:** Echinococcosis is a disease that affects large proportion of humans around the world due to exposure to the larvae of the *Echinococcus* spp tapeworms. The disease overwhelms surgeons especially in Iraq due to high numbers of cases. The disease control according to the World Health Organization (WHO) is costing the world billions of US dollars annually. **Objectives:** The current database information about the hydatid cyst (HC) phylogeny in Iraq is obscure, therefore the present work was conducted to identify the current circulating strains of *E. granulosus* in Iraq and their genetic links to world strains. **Patients and methods:** From Al Karama Teaching Hospital, Kut, Wasit, Iraq, 10 HC samples were collected from 10 patients who undergone surgical interventions to remove the cysts. The samples were investigated using polymerase chain reaction (PCR) and partial gene sequencing (PGS) techniques that targeted the mitochondrial cytochrome c oxidase subunit I (*COX*1) gene of the HCs. **Results:** The PCR findings confirmed that the HCs belonged to *E.granulosus*. Like the PCR, PGS assured the HC identity of *E.granulosus* plus declared that some local samples were in high nucleotide similarity with strains from the western, eastern, and middle eastern regions of the world. **Conclusions:** Thecirculating local strains in the sampled region of Iraq represent close similarity to some world strains referring to virulent potential or geographical links between these isolates.

Key words: COX1, Echinococcus, hydatid cyst.

Introduction

The zoonotic larval infection named as Echinococcosis is a global infection of the human being. This illness is triggered by the parasite *Echinococcus* spp. The global burden of disease management is over USD three billion per year, the WHO says. Hydatidosis is the outcome of a tapeworm (cestode) larval infection of *Echinococcus granulosus*. The characteristic of the disease is the growth of hydatid cysts (metacestode) within intermediate hosts' internal organs, including humans. Carnivores such as dogs are the definitive hosts of the cestode. People and other hosts accidentally uptake eggs or gravid proglottids which are released in the feces of the definitive host, causing the disease. Because of the pathogen 's nature, hydatidosis is more prevalent in populations where dogs are kept to protect and herd cattle. Regionally, the illness has a greater occurrence in worldwide distributions in the Mediterranean, Russia, China, North and East Africa, South America, and Australia (McManus *et al.*, 2003; Moro and Schantz, 2009; Deplazes *et al.*, 2017; Pourseif *et al.*, 2018).

When eggs are ingested, a larval oncospheres are liberated from the egg that may invade-penetrate the intestinal lamina propria. Then, it is passively transported to the liver, lungs, or other internal organs via blood or lymphatic stream, in which they grow into hydatid cysts (larvae of metacestodes). These cysts have an internal germinal layer and an external laminated layer, enveloped by a host-derived fibrous capsule. Smaller cysts called as "daughter" grow from the internal cellular layer. Cysts grow slowly in humans, and can reach in their volume up to multiple liters with thousands of protoscolices in their contents. Septations and daughter cysts gradually interrupt the typical unilocular structure of the cysts. Using the light microscopy, the HCs can be recognized by three different features of thick, acellular, laminated layer that hasa unique acidophilic staining activity, internal germinal layer (cellular), brood capsules or protoscolices, and a host-generated outerfibrotic layer (Li *et al.*, 2008; Wen *et al.*, 2019).

Hosts compromised can remain without displaying signs or symptoms of disease for several months or years. Certain patients have recurrent HCs for years without warning, some HCs may destroy accidentally or as a consequence of trauma and eventually vanish. If a HC remains to grow, the HC pressure on surrounding tissue may cause patients to develop illnesses incrementally. Immediate signs and symptoms are related to the spontaneous rupture more than cyst development. Cyst rupture may stimulate the IgE mediated response of hypersensitivity in the affected people, such as hives, swelling, and flushing. The reaction may present a danger to life. Ruptured or leaked cysts could cause secondary hydatidosis due to the presence of free protoscolices that affects the peritoneum. The type of signs and symptoms symptoms vary relying on the location of the HCs. The HCs are frequently prevalent in the liver (65%) and lungs (25%). Bones, spleen, brain, and cardiac muscles may also be affected. The cardiac symptoms vary according to the area. About 20 to 40 percent of patients are involved in multiple organ conditions. HC frequent symptoms involve pain in the abdomen, reduced appetite, hepatomegaly, mass on palpation, distention of the abdomen. Chronic cough, chest pain, and shortness of breath are typical symptoms in lung HCs (Almulhim and John, 2020).

Iraq is considered as hotspot for the disease due to the use of untreated guard and herd dogs and due to the presence of high numbers of stray dogs that may get their infections from eating contaminated meat or meat from infected dead animals. The current database information about the HC phylogeny in Iraq is obscure, therefore the present work was conducted to identify the current circulating strains of *E.granulosus* in Iraq and their genetic links to world strains.

Patients and methods

Surgical based collection of the samples

From Al Karama Teaching Hospital, Kut, Wasit, Iraq, 10 HC samples were collected from 10 patients who undergone scolicidalbased surgical interventions to remove the cysts (Sozuer, Akyuz and Akbulut, 2014). The samples were then transported to the processing laboratory for performing the PCR tests and sending out the PCR positive products to the sequencing facility.

DNA extraction, PCR, and partial *COX*1 sequencing

The DNA extraction from the aspirated HC fluids was conducted using gSYAN DNA Extraction Kit (Geneaid, Taiwan). The steps of the extraction belong to the kit were followed. The collected DNA via the extraction procedures was evaluated using a Nano Drop.

The primers used for the present investigation involved using the *COX*1 gene (at 450bp) as a target for performing the PCR analysis. The primers were F: TTTTTTGGGCATCCTGAGGTTTAT and R: TAAAGAAAGAACATAATGAAA ATG. The Maxime PCR PreMix Kit, in accordance with its instructions, was employed to prepare the PCR master mix solutions. DNA template at 5-50ng, each direction of the primer at (5µl), 10pmol (1µl), PCR water at 13µl for completing the total volume of the reaction, 20µl. The mixture was added to aPreMix Kit tubes that contained components such as DNA polymerase, dNTPs, KCl, $MgCl_2$, and a tracking dye. After brief mixing of the final mixture, the tubes containing the PCR reaction mixtures were inserted into athermocycler (BioRad. USA). The conditions of the cycler were a one-cycle-initial denaturation (94˚C) for 5min, followed by a 35-cycle-step of (denaturation (94˚C), annealing (50˚C), and extension (72˚C) for 45s/each step), and a one-cycle-final extension (72˚C) for 7mins. A 1.5%-agarose-gel preloaded with ethidium bromide dye plus 100-volts, 80-amps, and for-one-hour conditions were followed to examine the PCR products that were finally visualized using a UV-imager.

A PGS technique that targeted the *COX*1) gene was employed to analyze the HCs.

Results

The PCR findings confirmed that the HCs belonged to *E.granulosus*. The results were confirmed via the amplification of the tested genetic region of the *COX*1 gene at 450bp. The findings are revealed in the figure 1.

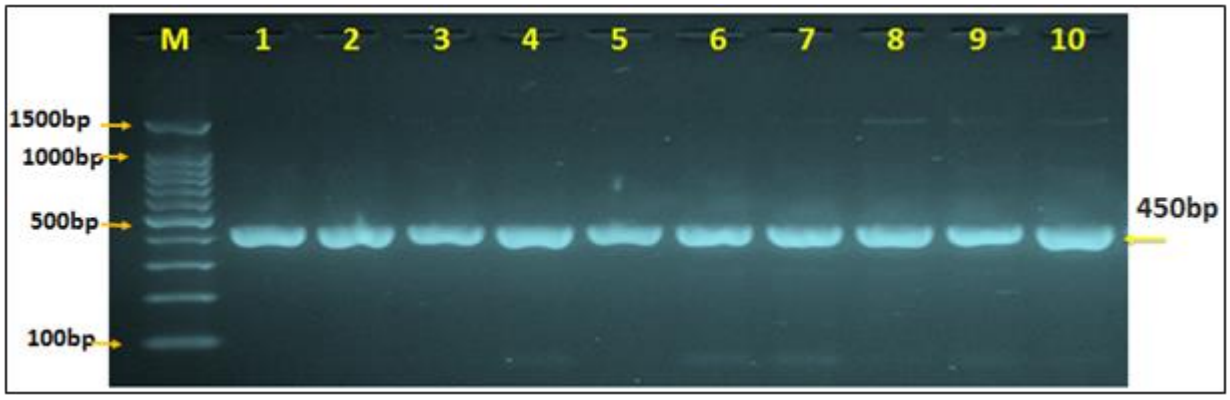

Figure 1: Agarose gel electrophoresis image of the *Echinococcusgranulosus* of the mitochondrial cytochrome (COX1) gene that belongs to the human-sample-isolated hydatid cyst (HC) fluids. M: Ladder, 1500-100bp, lanes 1 to 10:positive HC with*COX*1 gene at 450bpof size.

Like the PCR, PGS assured the HC identity of *E.granulosus* plus declared that some local samples were in high nucleotide similarity with strains from the western and middle eastern worlds. These analyses are displayed in figure 2 and 3.

Figure 2: Multiple sequence alignment analysis of local Echinococcusgranulosushydatid cyst fluids via the use of mitochondrial cytochrome c oxidase subunit 1 (cox1) partial sequencing. ClustalWsequence alignment tool (MEGA X version) were employed. Similarities are revealed, here, between the local and some world isolates.

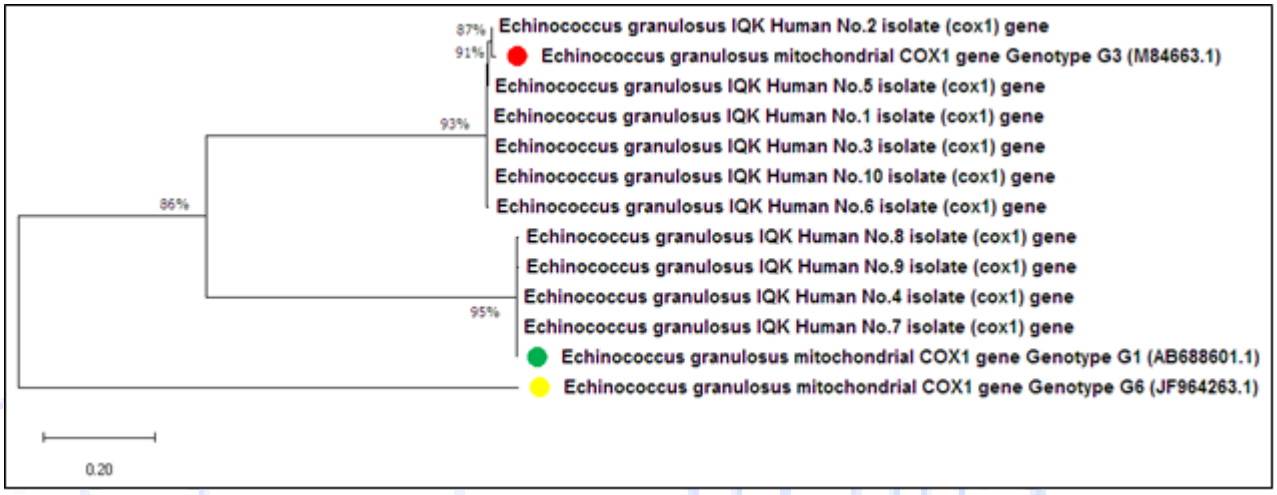

Figure 3: Phylogenetic tree of the Echinococcusgranulosushydatid cyst via the mitochondrial Cox1 gene partial sequencing. Maximum Composite Likelihood method (UPGMA tree) in (MEGA 6.0 version) was followed to calculate the evolutionary distances, local isolates (No.4, No.7, No.8, and No.9) and (No.1, No.2, No.3, No.5, No.6, and No.10) were genetic-based close to AB688601.1. and M84663.1, respectively.

The genetic similarities, table 1, between the local isolates were identified to be close to two strains, one (M84663.1) from Europe, South America, Asia, and Africa isolated during the early of the 90's of the previous century (Bowles, Blair and McManus, 1992) and one (AB688601.1) from Jordon and Iran (Yanagida *et al.*, 2012).

Table 1: Homology sequence identity of local *E.granulosus*Human isolates and some global isolates.

***E. granulosus* isolate**	**Homology Sequence identity**	
	Accession number	**Identity (%)**
IQK. No.1	M84663.1	99.45
IQK. No.2	M84663.1	100
IQK. No.3	M84663.1	99.73
IQK. No.4	AB688601.1	99.50
IQK. No.5	M84663.1	99.45
IQK. No.6	M84663.1	98.91
IQK. No.7	AB688601.1	99.75
IQK. No.8	AB688601.1	99.50
IQK. No.9	AB688601.1	99.25
IQK. No.10	M84663.1	99.18

Discussion

Iraq is considered as hotspot for the infection with hydatidosis due to the spread of *E.granulosus* untreated dogs that are used for guarding people and animal herds as well as their presence in the Iraqi streets and yards as stray dogs(Molan and Saida, 1989; Molan, 1993; Saeed *et al.*, 2000; Hammad *et al.*, 2018; Heendeniya and Bogoch, 2018). This spread of disease induces not only patients with different severities of the disease but also overwhelming Iraqi economic status and medical personnel. For genetic tracking purposes, the current work was conducted that found links between the circulating stains of the HC strains and some of the world dominant isolates.

According to the genetic similarity links between the Iraqi isolates and some of the world strains identified in the present study, bidirectional explanation of the herein results can be explored. The global strain isolated in Europe, South America, Asia, and Africa indicates a wide spread of the genetic variant in the world which makes it easy to be discoverable almost anywhere in the world. This could be a rational that we detected local Iraqi isolates that represent genetic similarities close to this world dominant strain. The high genetic similarity indicates probable evolution of the local strain by which the might have been originated from this global isolate. This suggestion can be supported especially when a local Iraqi isolate showed a one-hundred-percent genetic similarity with the global strain hinting its infection circulation in Iraq. For the close genetic relation with the isolate from Jordon and Iran, the current local Iraqi isolates may represent similarity and evolution via the geographical connections between Iraq and its neighbors, Jordon and Iran. Dogs, as the final hosts, and food animals, as the intermediate hosts, can easily move through the borders of these countries enhancing the exchange of their strains between them. Thus, it could be easy to detect this strain in any of these countries and other neighbor countries (Chaâbane-Banaoues *et al.*, 2015; Ohiolei *et al.*, 2019).

For medical intervention, different surgical procedures can be used. Within endemic countries, the majority of surgeons favor conservative procedures especially if the cyst can be exposed safely. In order to avoid the leak of cyst contents into the surrounding tissue and peritoneum, the pericystic region and field are protected by pads saturated with a scolicidal material. The cyst is penetrated and vacuumed. The aspired fluid is clear, colorless and named rock water in uncomplicated cysts. The scolicidal agent is inserted in the cyst after a complete aspiration of the cyst fluid to avoid dilution of the scolicidal agent. The scolicidal agent is then injected into the cyst interior space and left for around 5 to 15 minutes. The scolicidal agents currently used in such intervention are hypertonic saline (3%–30%), chlorhexidine, ethyl alcohol (70%–95%), cetrimide (0.5%), povidone iodine (10%),silver nitrate (0.5%), and hydrogen peroxide (3%).

In conclusion, the circulating local strains in the sampled region of Iraq represent close similarity to some world strains referring to virulent potential or geographical links between these isolates.

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