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The relationship between genetic diversity and fertility status of hydatid cysts of *Echinococcus granulosus* isolated from human and some intermediate hosts in Thi-Qar province / Iraq

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Abstract---The cestod *Echinococcus granulosus* produces hydatid cysts, which are an endemic zoonotic in Iraq. This study was conducted to assess the common strains and the effect of haplotype diversity on the fertility status isolated of hydatid cysts from livers of humans and other different organs of intermediate hosts (sheep, cattle, buffalo, and camels), by three mitochondrial DNA genes, including *cox1, nad1* and *atp6* extracted from the fertile cysts protoscolices and sterile cysts germinal layer. The result indicated that the sheep (G1 ,95%) and buffalo (G3, 5%) strains were responsible for the hydatid cyst infection. All isolates were recorded in the Genbank with numbers ranging from (OL655428- OL655447) for *cox1,* (OM117505-OM 117524) for *nad1*and (OM117525-OM117544) for *atp6*. The current study revealed windingly that no correlation between the haplotype of parasite and the sterility of hydatid cysts. Thus, our finding indicated that environmental factors might play a key role in the fertility of hydatid cysts.

Keyword---E.granulosus, Hydatid cysts, Cystic Echinococcos(CE), Haplotypes, Sterile.

Introduction

Echinococcosis or Hydatid disease , caused by the larvae of *E. granulosus* is the most serious tapeworm infection occurring in human. The lack of chemotherapy effectiveness and the surgical removing difficulties may be a reason of it spoor prognosis (Dybicz,2019; Khademvatan,2019). The larvae of this worm can infect in a wide range of intermediate host species, including domestic animals . This adaptation has given a worldwide distribution (Hama *et al* ., 2015). The life cycle of this parasite is complicated. The adult worm is attached to the small intestine wall of the dogs or other carnivores, while , the larval cysts (hydatid cysts) are found in different organs of domesticated and wild herbivores. Human represents an accidental intermediate host (Romig *et al.,* 2017), because of that its became a zoonotic disease and has economic importance in every continent (Thompson,2017).

The most common causal agent CE is *E. granulosus s. s* (genotypes G1 and G3) (Alvarez Rojas *et al.,* 2014). This species is found all across the world, especially in rural livestock-raising areas. South America, the Mediterranean basin, and Asia all have highly endemic foci (Deplazes *et al.,* 2017) . the sheep strain (G1) is the most common strain worldwide, and the hydatid cysts resulting from it are often fertile, and most cases of human infections are caused by this strain(Cucher *et al.,* 2016), as it is responsible for the vast majority of infections , this accounts for approximately 88% of cases worldwide (Higuita *et al.,* 2016). Hydatid cysts are divided into two types: fertile cysts and sterile cysts that do not generate protoscolices for unexplained reasons, therefore ending the parasite's life cycle. (Zhang *et al.*, 2003 ; Daryani *etal.*,2009), The mechanics behind the processes that lead to fertile or sterile cysts are unknown. (Paredes *et al.*,2007).

Although several studies have been conducted to better understand the genetic diversity of *E. granulosus,* the biological and molecular basis underlying the generation of both types of cysts, as well as the molecular mechanism involved in hydatid cyst fertility/sterility, remain not well understood and unknown (Vatankhah *et al.*,2003 ; Cabrera *et al.,*2008; Wassermann *et al.,* 2016; Kinkar *et al.,* 2017 ; Laurimäe *et al.,* 2018). immunological factors (IgG1) , as well as oxidative DNA damage caused by internal environmental factors , have been suggested played a role in hydatid cyst sterility(Cabrera *et al.,* 2008; Riesle *et al.,* 2014) .While Fallah *et al.* (2021) referred to it's likely that the host plays a key role in hydatid cyst fertility. This study was designed for the first time in Iraq to determine the *E. granulosus* strains genotypes, and to estimate the genetic variability and haplotyping within the strains, and shed the light on the more details about haplotype diversity and their effect on the fertility status of hydatid cysts using three genes (*cox1, nad1,and atp6*).

Samples collection

A total of twenty hydatid cysts samples were collected during the period from January to June 2021. Sixteen of these samples were obtained from Nasiriya slaughterhouses, including 6 from livers sheep, 6 from cattle (3 livers and 3 lungs), 2 from buffalo (liver and lung),and 2 from camel (lung and spleen). In addition, four human samples were livers infected with hydatid cysts which were collected from Al-Amal private hospital in Thi-Qar province. The hydatid cyst contents (protoscolices , germinal layers, and fluid), were then centrifuged and examined to confirm the fertilization status according to Smyth & Barrett(1980) and Macpherson(1985). Fertile cysts were determination under light microscopy, by the presence of grown Protoscolices). The rest of the sedimentation was then stored at -20 °C until DNA extraction (Esfedan *et al.,* 2018) .

DNA Extraction

DNA was extracted from protoscolices and germinal layer following the method attached to the kit ®Genomic DNA Purification Kit. (USA) and preserved at -20 °C until later use.

Polymerase Chain Reaction (PCR)

For the amplification of the *mt cox1, nad1, and atp6* genes of extracted DNA samples , PCR technique was used.

Table 1
Shows the used primers

	Target gene Primer Name	Primer sequence (5`to 3`)	Tm ©	Product Size (bp)	Ref.
	mt cox1 (*Cytochrome oxidasesubunit 1*)	F . (EgCOI1) TTTTTTGGCCATCCTGAGGTTTAT	56	450	Bart *et al.,*2006)
		R . (EgCOI2) TAACGACATAACATAATGAAAATG	**56**		
	mt nad1 (NADH) (400bp) dehydrogenase subunit 1	F. (MS1) CGTAGGTATGTTGGTTTGTTTGGT	50	400	(Sharbatkhri *etal.,* 2009)
		R. (MS2) CCATAATCAAATGGCGTACGAT	50		
	mt atp6) ATPase subunit 6	F. (AT1) TTGAAGCGTTGGAGATAA	60	848	(Rostomi Nejad *et al.,*2012)
		R. (AT2) CAGACGATAACCCAGACAT	60		

Each PCR reaction was carried out in a final volume of 20 µl by adding 1 µl of forward and reverse primer, 5 µl of the genomic DNA, and 13 µl of nuclease-free water from Bioneer Accupower® PCR PreMix. The reaction was executed out in thermocycler under the following conditions for all genes : a pre-denaturation step at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 56 ,50 , 60 °C for *mt cox1, nad1, atp6* genes respectively for 45 sec and extension at 72 °C for 45sec plus a final extension step at 72 °C for 7 min. DNA samples were loaded into the appropriate wells of the TAE agarose gel (1.5% (w/v)) .Sample stained with 3 µl ethidium bromide dye. The agarose gel was run at 100V for 60 minutes. The DNA within the agarose gel was visualized using a UV transilluminator.

DNA Sequencin

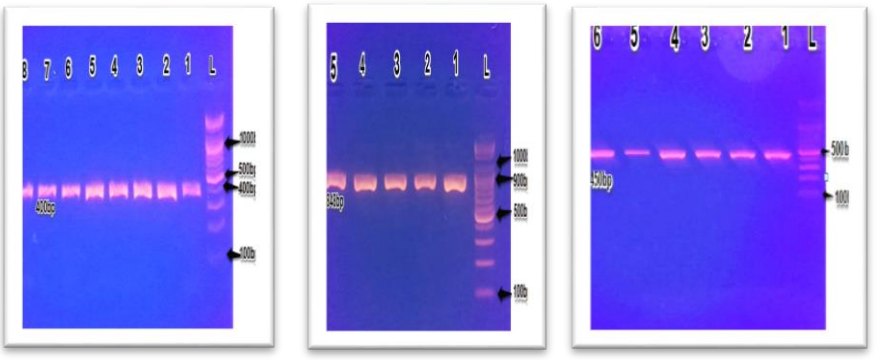
The PCR products obtained as described above were purified utilizing the Geneaid DNA Cleanup kit . Nucleotide sequencing of the purified PCR products was done in both directions using the forward *mt cox1, nad1*,and, *atp6* genes. The sequencing was done by a South Korean public biotechnology company (Macrogen). Genetic sequences results were then corrected in a BioEdiet sequence alignment editor v.7.2.5 program**.** The sequences of hydatid cysts samples were then recorded in The National Centre for Biotechnology Information Service (NCBI, https://www.ncbi.nlm.nih.gov/) and the accession numbers were assigned for these sequences.

Phylogenetic tree for the strains

Phylogenetic tree analysis was performed using genetic distance, which was determined by the Maximum Composite Likelihood method, the UPGMA method and by MEGA-X software v.10.0.4, according to Kumar (2018).

Results

Using mitochondrial primers (mentioned above), DNA was isolated from Protoscolices or germinal layer of 20 hydatid cysts samples for molecular investigation to confirm the presence of these genes. This study found that the partial sequencing of *cox1 , nad1 , atp6* genes produced ≤ 450bp , ≤ 400 bp ≤ 848 bp, respectively for each samples obtained from the selected hosts (Figure 1).



A B C

Figure 1. Agarose gel electrophoresis of PCR amplification (A= *cox1* , B=*nad1* and , C=*atp6*) using 1.5% agarose, 100 V, for 1 hrs. (L: 1000 bp DNA ladder , lanes 1-5: contain PCR products of hydatid cysts isolates)

Sequence identical ratio was also recorded with other samples in NCBI-BLAST , 100 % for *cox1* gene , while was the ratio between 100%- 99.73 for *nad1*gene , and 100%-99.88 for *atp6* gene. The obtained sequences were deposited in the Genbank database under the accession numbers from OL655428 - OL655447 for *cox1,* OM117505 - OM117524 for *nad1,* and OM117525 - OM117544 for *atp6.* Table (2) shows the sheep strain G1 is dominant, in high proportion, followed by the buffalo strain (G3) .

Table 2
Genotype of the isolates of *E .granulosus* strains from intermediate hosts and organs isolates from both fertile , and sterile cysts

N	Host	Organ	Fertility	Genotype	N	Host	Organ	Fertility	Genotype
1	Sheep	liver	Fertile	G1	11	Cattle	lung	Sterile	G1
2	Sheep	liver	Fertile	G3	12	Cattle	lung	Fertile	G1
3	Sheep	liver	Sterile	G1	13	Cattle	lung	Fertile	G1
4	Sheep	liver	Sterile	G1	14	Sheep	liver	Fertile	G1
5	Sheep	liver	Fertile	G1	15	Cattle	liver	Fertile	G1
6	Human	liver	Fertile	G1	16	Buffalo	liver	Sterile	G1
7	Human	liver	Fertile	G1	17	Buffalo	lung	Fertile	G1
8	Human	liver	Sterile	G1	18	Camel	lung	Sterile	G1
9	Human	liver	Sterile	G1	19	Cattle	liver	Sterile	G1
10	Cattle	liver	Fertile	G1	20	Camel	spleen	Sterile	G1

This study found that nine sterile samples were found in different haplotypes (Table 3), and different clusters in phylogenetic tree (Figure 2). We have found seventeen haplotypes from 20 samples. A single haplotype was found in 15 samples of them, whereas, the rest (5 samples) were found in two clusters (Table 3).

Table 3
Haplotype frequencies in populations for study samples depending all genes study

N	Samples	Haplotype	Haplotype frequencies in populations	Relative frequencies %
1	1	H1	1	5
2	3	H2	1	5
3	4	H3	8	5
4	5	H4	1	5
5	8, 14	H5	2	10
6	7	H6	1	5
7	6	H7	1	5
8	16	H8	1	5
9	20	H9	1	5
10	17	H10	1	5
11	18	H11	1	5
12	9	H12	1	5
13	10	H13	1	5
14	11	H14	1	5
15	12 , 13, 19	H15	3	15
16	15	H16	1	5
17	2	H17	1	5
	Gene diversity / mistake percentage	0.9789 0.0245 +/-	20	100

Figure (2) showed the haplotypes in four clusters, consisting from different numbers of samples. In contrast, three single haplotypes were also observed. The samples (12, 13, 19) as well as (8, 14) were seen as completely identical, whereas, the rest sample were not (Table 3 & Figure 2). This study found that 95% of sample were sheep strain (G1), and only 5% of them was from buffalo strain (G3).

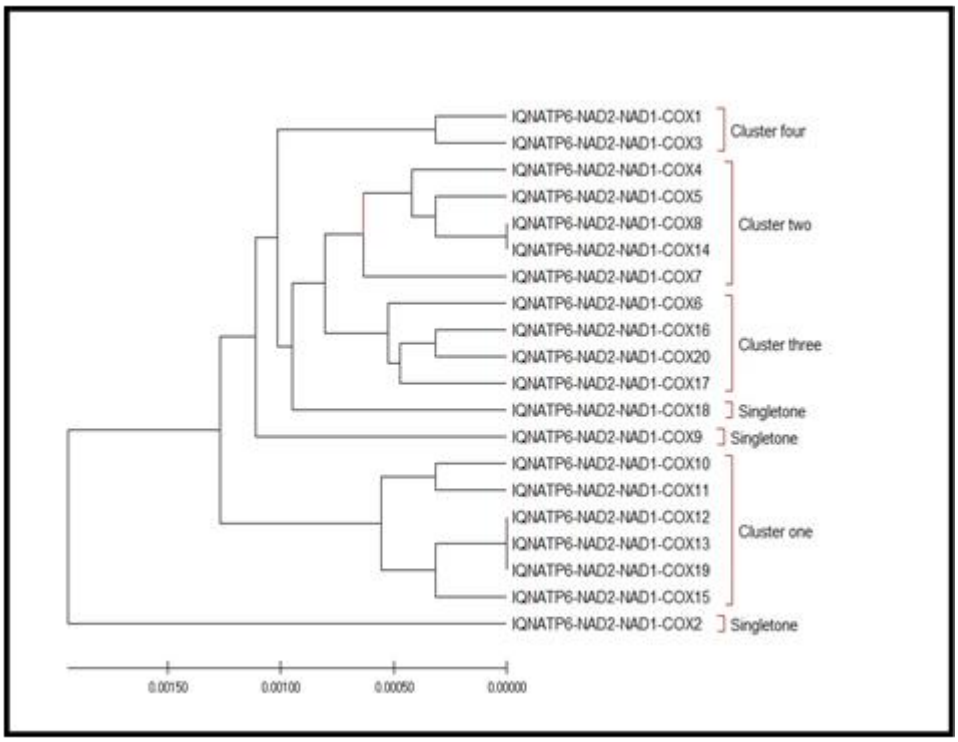


Figure 2. Phylogenetic tree analysis by maximum likelihood method depending all genes study

Discussion

The results showed that the dominance strain was a sheep strain (G1 ,95%), whereas, only 5% of samples were buffalo strain (G3). These strains may be a cause of hydatid cyst infections in Thi- Qar province. This result agreed with previous studies in Iraq based on the polymerase chain reaction (PCR) and use both *cox1, nad1*, or one of them, (Hanash , 2016 ; Hammad *et al* ., 2018 ; Lazim , 2019 ; Al-Rishawi, 2019 ; Al-Sadi *et al.*, 2021 and AL-Ataby ,2022) . However , the present results disagreed with the studies of (Al-Shimary,2019; AL-Yacoubi , 2021), Were recorded G1, G2, G3 , and G5. Taken together, these findings confirm that the most infected strain of *Echinococcus* species word wide is a sheep strain (G1). Because, can infect a variety of hosts (Alvarez Rojas *et al.,*2014; Thompson, 2017). Although, the dominating G1 and G3 strains in Iraq , high diversity haplotypes were recorded (17 haplotypes) as shown in (Table 3& Figure 2). This gave a clear picture of the genetic diversity within the single genotype in the study area, this significant diversity in the individual patterns may be result of the wildlife hosts and their interaction with the domestic hosts . (Haag *et al.,* 2008; Nakao *et al.,* 2009; Nakao *et al.,* 2010; Nakao *et al.,* 2013b). Furthermore, the final host might be infected by different genetically adult worms, that reproduce by cross & self-fertilization, and in turn produce varity of eggs that have different genetically cysts . (Ziadinov *et al.,* 2010; Lymbery, 2017).

Through our observations of the phylogenetic tree and genetic diversity (Figrue 2), and (Table 2 ,Table 3) for the nine sterile isolates (3, 4, 8, 9, 11, 16, 18, 19 and 20), we did not recognize an assembly in one genetic distance for these sterile cysts, as well as in the fertile isolates (1, 2, 5, 6,7 ,10, 12, 13,14, 15, 17). On the contrary, the sterile cysts isolates distributed were found in all genetic groups (Cluster) and in as single haplotypes along the phylogenetic tree in some cases, a complete identical was found between sterile, fertile isolates. Consequently, there is no clear effect of haplotypes on the fertility or sterility of the cysts, meaning that the fertility stuts of the protoscolices are not a genetic trait. This disagree with the suggestion of Fallah *et al.* (2021), who indicated to the role of genetic effects on fertility of cysts . Our study supported the previous studies that indicated to the immunological factors (IgG1) and the oxidative damage of DNA produced by reactive oxygen and nitrogen species and nitrogen free radicals as a results of internal environmental factors may play a role in stimulates the sterility of hydatid cysts (Cabrera *et al* 2008; Riesle *et al* ., 2014).

Conclusions

The result concludes that sheep strain(G1) is dominant strain, followed by the buffalo strain (G3) of *E. granulosus* in Thi-Qar province. In addition, there is no effect of genetic traits (Haplotype) on hydatid cyst sterility. Environmental factors might be the cause of hydatid cyst sterility, so that further study is required

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