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MOLECULAR DETECTION OF *ECHINOCOCCUS GRANULOSUS* STRAINS OF HUMAN HYDATIDOSIS IN AL-MUTHANA PROVINCE

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Abstract

Ten samples of human hydatid cysts samples were collected from Al Hussein Educational Hospital and Al-Khider General Hospital in Al-Muthana province in central Iraq from July 2018 to May 2019. The study aimed to molecular identification of the common strains of *Echinococcus granulosus* parasites in the province of Muthana using PCR technique, as well as through the study of the gene sequence of the strains and the drawing of the phylogenetic tree to determine the most common strains in human in the province. The sources of hydatid cysts in all current study samples were the liver only. DNA was extracted from the germinal layer in the hydatid cysts and then the product was amplified by PCR technique by Touchdown method where the mitochondrial gene cox1 was targeted with a molecular weight of 450 base pairs. The results were positive in nine samples and negative in only one. The results showed that the prevalence of infection in females is higher than in males 80% and 20%, respectively. The most affected group was the young age group 16-30 years, with 50% ratio also showed the prevalence rate in the rural population was the highest 70% than urban 30%. The results of the sequencing of gene cox1 and the phylogenetic tree analysis its comparison with the isolated of *E. granulosus* in the gene bank showed that the common to human pathogenic strains in Al-Muthana province were the buffalo strain (G3) (60%) followed by the sheep strain (G1) in second place (40%).

Key words: Hydatidosis, Echinococcus, G1, G3, Genotype, Al-Muthana, Iraq

Introduction

Cystic echinococcosis (CE) is a common zoonosis disease that has not been sufficiently addressed despite its significant effect, it's caused by the larval stage of *Echinococcus granulosus*, also its an endemic disease on all continents except Antarctica (Otero-Abad & Torgerson, 2013), CE remains a public health problem in developing countries and has the highest prevalence of disease in humans in rural sheep breeding communities whereas sheep are the most important intermediate hosts (Abdulhameed *et al*., 2019).

Hydatid cystic disease has been known since ancient times and are one of the most serious epidemics that harm humans both in terms of health and economics (WHO, 2018). According to The World Health Organization (WHO), the Current estimates that cystic echinococcosis results in the loss of 1–3 million disability-adjusted life years (DALYs) annually. Annual costs associated with cystic echinococcosis are estimated to be US$ 3 billion for treating cases and of losses to the livestock industry.

The disease causes 19,300 deaths worldwide annually, WHO classifies it among 17 neglected tropical diseases (NTDs) (Higuita *et al*., 2016).

In Iraq, cystic echinococcosis is one of the main health concerns (CDC, 2012), which is endemic and a major health problem in the country, It is even more complicated because of that CE didn't have a real systematic national surveillance and control program in Iraq (Athmar & Ban-Abbas, 2014). The largest number of human cases were reported in the central and southern governorates of Iraq including Basrah, Dhi Qar and Al-Muthana (Abdulhameed *et al*., 2019).

Molecular studies have indicated that ten genotypes or distinct Strains for *Echinococcus granulosus* sensu lato differ in epidemiology, pathogenesis, infection, control, external appearance, intermediate and pathological species, geographical distribution, pupae, larvae and other traits (Eckert & Thompson 1997). The genetic patterns of granulocytes have been determined on the basis of molecular genetic analysis using DNA sequence, Phenotype and Gene Sequences. These strains are *E. granulosus* sensu stricto, which includes genotypes (G1-G3) *Echinococcus equinus* or horse strain (G4) and *Echinococcus ortleppi* (G5) the cattle strain and *Echinococcus canadensis*, which includes the G6-G10 strains (Romig *et al*., 2015). The aims of the study are to determine the main strains of human hydatidosis in Al-Muthana province, study of gene sequences of these strains and the phylogenetic tree analysis by cox1 gene.

Materials and Methods

Samples Collection

Ten samples of human hydatid cysts were collected from July 2018 until May 2019. We have it after completed of surgery for patients with CE from two hospitals in Al-Muthana province (eight of which were collected from Al Hussein Educational Hospital in the Samawah city and two from Al Khider General Hospital in Al-Khider district). These samples were stored in formalin solution and kept under deep freeze cooling until they were transferred to the molecular laboratory to complete subsequent operations. Small pieces from the germinal layer of the hydatid cysts take and placed them inside the test tubes to the next step. The tubes are centrifuged at 3000 rpm 15 minutes. The remaining liquid is poured at the end of time. The remaining pieces in the test tubes then washed with 10 mL of normal saline solution. The cycle is repeated at the same speed and duration for purification of the isolations (Al Azawi *et al*. 2014). Then the cut-off parts of the germinal layer are then stored with 70% ethanol and kept under deep freeze at -20 °C until DNA extraction is performed in the molecular laboratory (Esfedan *et al*., 2018).

PCR technique was performed for in detection and genotyping of *Echinococcus granulosus* hydatid cyst based on mitochondrial Cox1 gene (450 bp) in isolates. This

technique was carried out according to the method described by (Nikmanesh *et al*., 2014) as the following steps:

Genomic DNA Extraction

Genomic DNA from hydatid cyst germinal layer for each sample was extracted by using gSYAN DNA Extraction Kit (Geneaid Taiwan), and done according to company instructions as following: A 200µl of frozen hydatid cyst germinal layer was transferred to a sterile 1.5ml microcentrifuge tube and placed in the centrifuge at 10000rpm for 1 minute. The supernatants were discarded and added 200µl GST buffer and 20µl of proteinase K and mixed. Then incubated at 60℃ for 15 minutes. After that, 200µl of GSB buffer was added and mixed then all tubes were incubated at 60℃ for 10 minutes and inverted every 3 minute. Then 200µl of absolute ethanol were added to the lysate and immediately mixed vigorously. DNA filter column was placed in a 2 ml collection tube. Then centrifuged at 10000rpm for 5 minutes, the 2 ml collection tube containing the flow-through was discarded and placed the column in a new 2 ml collection tube. After that 400µl of W1 buffer was added to the DNA filter column, then centrifuged at 10000rpm for 30 seconds. The flow-through was discarded and placed the column back in the 2 ml collection tube. Then 600µl of Wash Buffer (ethanol) was added to each column. Then centrifuged at 10000rpm for 30 seconds. The flow-through was discarded. All the tubes were centrifuged again for 3 minutes at 10000 rpm. The dried DNA filter column was transferred to a new 1.5 ml microcentrifuge tube and 50 µl of pre-heated elution buffer was added to the center of the column matrix. The tubes were let stand for at least 5 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 30 seconds to elute the purified DNA.

Genomic DNA examination

The extracted germinal layer genomic DNA was checked by using Nanodrop spectrophotometer (THERMO.USA), which measured DNA concentration (ng/µL) and check the DNA purity by reading the absorbance at (260 /280 nm).

Primers

PCR primers were used in this study for detection and Genotyping of *Echinococcus granulosus* based on sequencing of Mitochondrial cytochrome cox1 genes that amplification by using the PCR technique. These primers were designed (Nikmanesh *et al*., 2014) and provided by (Macrogen company, Korea) as the following in table (1).

Table 1 : PCR primers were used in this study

Primer		Sequence	Amplicon
cox1 gene	F	TTTTTTGGGCATCCTGAGGTTTAT 5'-3'	450bp
	R	TAAAGAAAGAACATAATGAAAATG 5'-3'	

PCR master mix preparation

PCR master mix was prepared by using (Maxime PCR PreMix Kit) and this master mix has done according to company instructions as following: 5µl of DNA template, 1 µl of cox1 gene forward primer, 1 µl of cox1 gene reveres primer and 13 µl of PCR water.

After that, these PCR master mix component that mentioned in the table above placed in standard PCR PreMix Kit that containing all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, $MgCl_2$, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000 rpm for 3 minutes. Then placed in PCR Thermocycler (BioRad.USA).

PCR Thermocycler Conditions

PCR thermocycler conditions were done by using conventional PCR thermocycler system as following steps: the Initial Denaturation at 94 ºC for 5 minute and one cycle, (Denaturation at 94 ºC for 45 second, Annealing at 50 ºC for 45 second, Extension 72 ºC for 45 second) these 3 steps repeated 35 cycle, then the final extension at 72 ºC for 7 minute and one cycle, finally the hold steps at 4 ºC.

PCR product analysis

The PCR products of mitochondrial genes were analyzed by agarose gel electrophoresis following: 1% Agarose gel was prepared in using 1X TBE and dissolving in the water bath at 100 ºC for 15 minutes, after that, left to cool 50 ºC. Then 3µl of ethidium bromide stain were added into the agarose gel solution. Agarose gel solution was poured in tray after fixed the comb in proper position after that, left to solidify for 15 minutes at room temperature, then the comb was removed gently from the tray and 10µl of PCR product were added in to each comb well and 5ul of (100bp Ladder) in one well. The gel tray was fixed in the electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 100 volts and 80 AM for 1hour. PCR products (450bp) cox1 gene was visualized by using UV transilluminator.

DNA sequencing method

DNA sequencing method was performed for genotyping of some positive local *Echinococcus granulosus* hydatid cysts isolates as the following step: The PCR product of mitochondrial Cox1 gene were sent to Macrogen Company in South Korea. The DNA sequencing analysis (Phylogenetic tree analysis) was done by Molecular Evolutionary Genetics Analysis version 6.0. (Mega 6.0) and Multiple sequence alignment analysis based Clustal alignment analysis and the evolutionary distances were computed using the Maximum Composite Likelihood method by phylogenetic tree UPGMA method. The identified *Echinococcus granulosus* genotypes isolates were submitted into of NCBI-GenBank.

Results

All the samples collecting from the liver of the patient. The prevalence of hydatid cysts according to the age, sex and the residence in of the patient. The results showed that the most affected age groups were 16-30 years of age the highest (50%), followed by 31-45 years (30%). the lowest is the group under 15 years and above 45 years by 10% each. According to sex, females were most affected than males, 80% and 20%, respectively. According to residence urban or rural areas, the highest prevalence was in rural areas (70% and 30% in urban areas as shown in Table (2).

Table 2 : Prevalence of hydatid cysts according to the age, sex and the residence in of the patient

Age group Year	No.	%	Sex				Residence			
			Male	%	Female	%	Urban	%	Rural	%
1-15	1	10	1	10	-	-	-	-	1	10
16-30	5	50	1	10	4	40	2	20	3	30
31–45	3	30	-	-	3	30	1	10	2	20
46-60	1	10	-	-	1	10	-	-	1	10
Total	10		2	20	8	80	3	30	7	70

DNA Extraction Results

The results of the DNA extraction showed that the concentration of samples was ranged from 1.9 to 36.2 ng/µl and the mean was 11.47 ng/µl and the results of DNA purity (OD260/ OD280) in the samples was ranged 1.07-1.34 and mean 1.16.

Results of PCR for the cox1 gene

The results of DNA amplification as showed in agarose gel electrophoresis positive samples in nine samples and one negative sample with number 6, as showed in figure (1). The results show the presence of the diagnostic mitochondrial cox1 genes for the *E. granulosus* was shown at 450 bp.

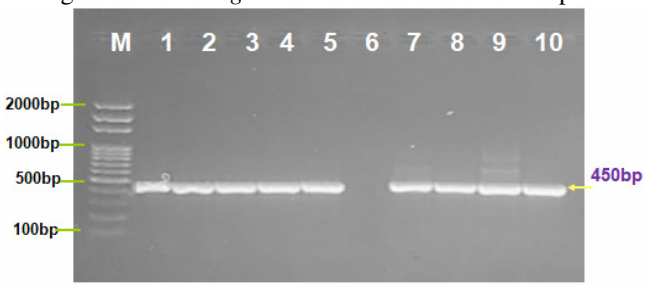

Fig. 1 : Agarose gel electrophoresis image has shown the PCR product analysis of cox1 gene in *E. granulosus*, Where M marker (2000-100bp), lane (1-5 and 7-10) positive cox1 gene at 450 bp PCR product, and one negative sample (6).

Results of DNA sequencer technique and Phylogenetic tree analysis

Results of *E. granulosus* Genotype

The results of the DNA analysis of a PCR product for five samples were randomly chosen of the specimens from the all ten samples its showed that sheep strain (G1) and buffalo strain (G3) is the common strains in human hydatid cyst in Al-Muthana province. The samples were sent to Macrogen Company in South Korea, which identifies the DNA sequence.

The results showed that the buffalo strain (G3) was present in 60% of the samples and 40% for the sheep strain (G1), as shown in Figure (2), (3) and table (3). The phylogenetic tree was constructed using The evolutionary distances were computed using the Maximum Composite Likelihood method (UPGMA tree) in (MEGA 6.0 version), the local Human *E. granulosus* human isolates (No.1) and (No.3) were show closed related to NCBI-Blast *E. granulosus* isolate (Genotype 1 : KT446001.1). The local *E. granulosus* human isolates (No.2, No.4, and No.5) were showed closed related to NCBI-Blast *E. granulosus* isolate (Genotype 3: M84663.1) at total genetic change (0.5-2.0).

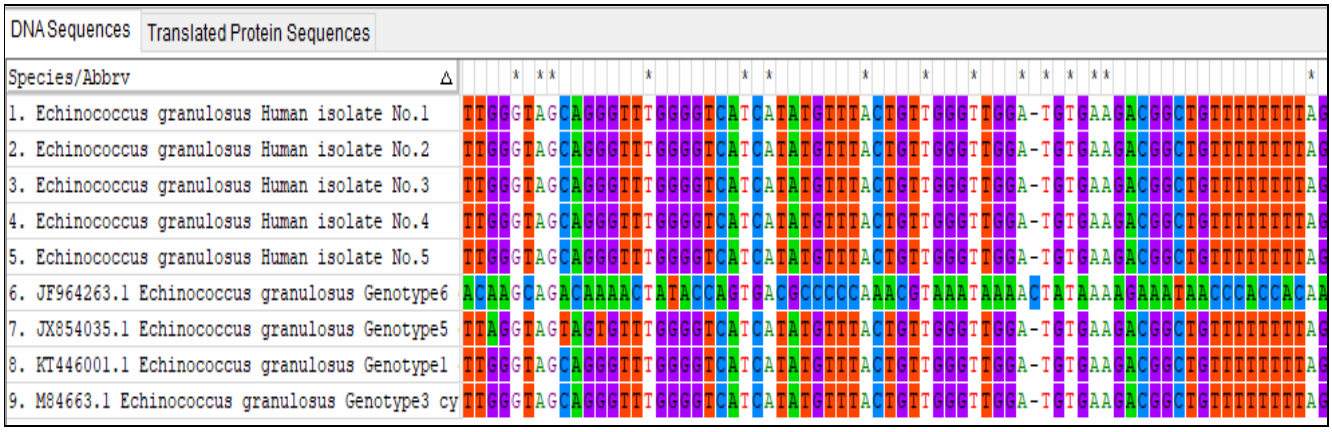

Fig. 2 : Multiple sequence alignment analysis of mitochondrial Cox1 gene partial sequence in local *Echinococcus granulosus* Human isolates with NCBI-BLAST *Echinococcus granulosus* related Genotypes isolates by using (MEGA 6.0, multiple alignment analysis tool). The multiple alignments the analysis was showed the similarity (*) and genetic variation (Substitution mutation) at mitochondrial Cox1 gene nucleotide sequences.

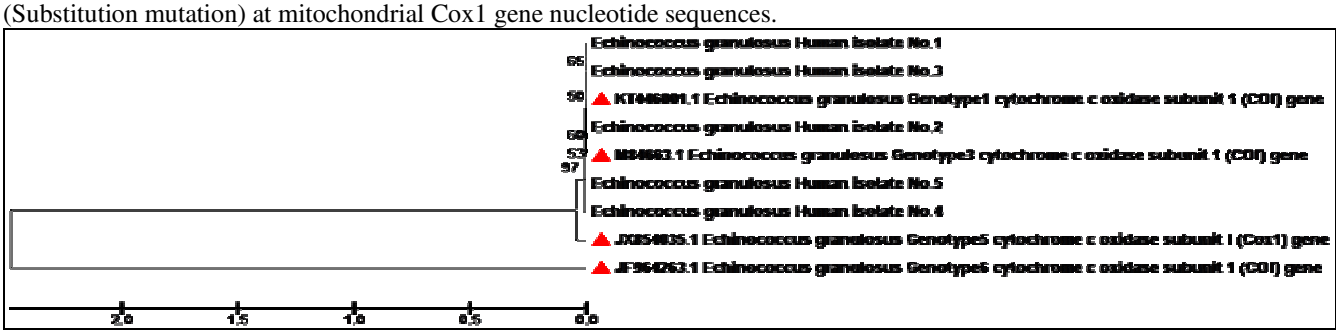

Fig. 3 : Phylogenetic tree analysis based on the Mitochondrial Cox1 gene partial sequence that used for *Echinococcus granulosus* typing detection.

Table 3 : NCBI -BLAST Homology sequence identity between local *Echinococcus granulosus* Human isolates with NCBI-BLAST *Echinococcus granulosus* related Genotypes isolates.

Local isolate	NCBI-BLAST Homology Sequence identity			
	NCBI BLAST identity isolate	Genotype	Accession number	Identity (%)
isolate No.1	*E. granulosus*	G1	KT446001.1	99.76%
isolate No.2	*E. granulosus*	G3	M84663.1	100%
isolate No.3	*E. granulosus*	G1	KT446001.1	99.76%
isolate No.4	*E. granulosus*	G3	M84663.1	99.16%
isolate No.5	*E. granulosus*	G3	M84663.1	99.72%

Discussion

All samples collected from the liver. The results showed that the age groups were 16-45 years are the highest (80%). Also, 80% of the patient were females and 20% males. Hydatid cysts were prevalence in al-Muthana province in rural areas 70% and 30% in urban areas.

The result agreed with Agha (2015), who show that the prevalence of hydatid cysts in human 91.66% in the liver, also its showed the female more affected than male 75% and 25%, respectively, the high infection 83.33% were in young group 16-45 years old. This study also agreed with Al-Yasari & Al-Shaiely (2013) and Rahi *et al*. (2015) who showed that the prevalence of hydatid cysts in females more than males and the prevalence of hydatid cysts in rural areas more than urban areas. The current study mismatched with Ahmed, (2013) where the majority of human infection in the lung 58.82%, while in the liver was the lowest rate 41.18%.

The results of the DNA concentration in this study indicated that the range was 1.9 - 36.2 ng/µl and the mean was 11.47 ng/µl. The results of DNA purity in the samples were 1.07-1.34 and mean 1.16 OD260/OD280. These results are matched with Ahmed *et al*. (2013), who showed that the range of concentration of the samples was 2.60-83.20 ng/µl while the purity range 0.1-2.12 OD260/OD280. The results were also consistent with Hammad (2017), which recorded the range of sample concentration was 1.01-16.37 ng/µl and the mean 6.47 DNA purity range of 0.75-4.50 OD260/ OD280.

Recently, the molecular identification method of *E. granulosus* strains is the most important way to obtain the correct information about taxonomic units to understand the epidemiology of common animal diseases and thereby develop appropriate control and control programs in preventing transmission and completion of their life cycle, In Iraq, molecular studies to determine the types and strains of *E. granulosus* are few (Ahmed *et al*., 2013; Hammad, 2017).

The results of the current study of DNA sequencing showed that the sheep strain (G1) and the buffaloes strain (G3) were the most common strains for human infections in Al-Muthana province where the (G3) strain was found in 60% of the samples and (G1) strain in 40% with a percentage of 99.76% matching the (KT446001.1) of (G1) strain and identity ratio of 99.16% and 100% of the (G3) strain with the corresponding sample labelled (M84663.1) in the gene bank.

The current study it's agreed with Agha (2015), which also found that the G1 and G3 strains are common in patients with hydatid cyst in Al-Qadisiyah province and in the same size as the sample it's showed that (G3) strain is the most common (60%) and (40%) for (G1) strain. In another study by Rahi & Ali (2016) conducted in the Al-Kut city, the results were identical to what we have recorded that the (G1) and (G3) strains are the common strains in the Al-Kut city among those infected with hydatid cyst, but differ in that the strain (G1) was The most common was 80% and the (G3) was only 20%. The present study also agreed with Busi *et al*. (2007) in Italy who showed in eight patients with hydatid cyst 87.5% of them were infected with (G3) strain and 12.5% were (G1) strain. Also agreed with a study of Piccoli *et al*. (2013) in Romania.

Pezeshki *et al*. (2013), Nikmanesh *et al*. (2014) and Khademvatan *et al*. (2019) in Iran They reached the that (G1) and (G3) strains are the most common strains in human infection, but (G1) the highest, also in Algeria Zait *et al*. (2016) and Brazil Mario *et al*. (2011). The results mismatched to another study in Iraq Hama *et al*. (2012) who detected G1 in all human samples where the result showed 100% matching to sheep strain (G1).

Conclusions

1. The prevalence of infection is highest in females 80% more than males 20%.
2. The most affected group was the age group 16-30 years.
3. The prevalence rate of hydatidosis in the rural areas was highest than urban areas.
4. The results showed that the (G3) strain is the most common strain in human followed by the sheep strain (G1) in Al-Muthana province.

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