Molecular investigation of *Echinococcus granulosus* in infected patients in Babylon province

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Abstract

The current study which was conducted during the period from (1/5/2022 to 12/31/2022) aimed to follow up people with echinococcosis in the hospitals of Babylon Governorate and some of its different districts, which included Hilla Surgical Hospital, Imam Ali Hospital, Al Hashimiya Hospital, in addition to (Al-Salam, Babylon , Al-Hayat) for the purpose of determining the prevalence of hydatid cyst worm by diagnosis by molecular investigation. The study included examining 94 blood samples distributed into **three** groups of **32** blood samples for people infected with echinococcosis before the hydatid cyst removal operation (12 males and 20 females). **32** blood samples for people after the surgical operation and removal of hydatid cysts and within (3-6) months (21 males and 11 females) and females) and **30** samples for the control group (healthiest) (17 males and 13 females). The clinical examination was conducted by Consultant doctors as well as diagnosis by | X-ray, ultrasonography, and tomography computer showed the results of the molecular study of hydatid cyst samples of infected people after extracting DNA from the germinal layer and protoscolices of hydatid fluid, using the multiplex polymerase chain reaction (PCR) technique and the tree phylogenetic tree. And based on the diagnostic gene (cytochrome oxidase Col) with a size of bp446 nucleotide pair, and comparing the similarity rates among the isolates registered globally and using the MEGA program, and comparing them with the similarity rates in our current study with the local and global isolates registered in the NCBI Global Gene Bank and within the base database and using the program NCBI where we were provided with the universal sequence numbers of the isolate .

Keywords : MEGA, NCBI, protoscolices, Hydatid.

Introduction

Hydatid disease is a widespread disease in all countries of the world, and due to the presence of large numbers of stray dogs infected with adult worms(**1**), it is considered one of the chronic diseases in humans due to the persistence of the cyst for long periods(**2**). The disease is known by many names, including Hydatidosis, Echinococcosis, or Echinococcosis Cystic (**3,4) .** Carnivorous carnivores are a definitive host to the parasite. The canine family includes Canine (dogs, hyenas, wolves, leopards, and some other ferocious animals), while animals are herbivores. Herbivorous such as (sheep, cows, camels, buffaloes, horses, etc.) as an intermediate host of the parasite (**5**) The disease affects the organs in which it resides in humans and the intermediate host alike primarily the liver and lung , spleen, brain, and other muscles except for hair and nail areas (**6**). Molecular investigation using the polymerase chain reaction method is one of the modern immunological methods for diagnosing parasitic infections and identifying Its ratio and location had an important impact in diagnosing the type and strain of the parasite ***E. granulosus,*** which causes cyst hydatid disease, due to its sensitivity and high specificity in diagnosing the parasite.molecular studies indicated that there are approximately 15 genotypes of the genus *Echinococcus* . *granulosus* (**7),** where

the genotypes or strains are called with the letter (G) for short, which is (G10-G1), and based on the analysis of the nucleotide sequence, the parasite *strain E. granulosus* was classified into several groups based on the genetic sequence, and some strains include the sheep. Sensu *E. granulosus* Strict genotype . (G3 G1), camel strain *Echinococcus Canadensis* with genotype (G6-G10)), horse strain *E.equinus* type G4, pig strain G7 pattern, and deer strain (G8-G9) (**8**)

Materials and Methods

Cyst collection group : Samples of cysts were collected through coordination with the doctors of hospitals in Babylon Governorate, by taking the sample from the operation room in a sterile plastic container and placing it in a refrigerated container until it is transported directly to the laboratory

Molecular study : Identification Molecular

PCR Reaction chain Polymerase

This technique was used in our current study to detect genotypes of *E. granulosus* from the protoscolices of human samples infected with the parasite. This technique was applied according to (**9**)

Primers:

The primers responsible for diagnosing the parasite *Echinococcus granulosu*s were obtained by targeting one of the mitochondrial genes. The primers were supplied by Canada IDT and as shown in the following table

Table (1) Primers used in the study with test results PCR

Primer	Gene name	Size
T T T T T T G G G C A F	Cox1	44bp
R T C C T G A GG T T T A T		

DNA extraction:

The DNA extraction process was carried out from the protoscolices by using the DNA Genomic Tissue kit prepared by the Turkish company Anatolia. Extraction was carried out according to the following instructions.

1 - 100 mg of the sample moved which is placed in Test tubes into the centrifuge at a speed of,1000 revolutions / min for a minute, to isolate the alcohol from the remaining precipitate from the protoscolices. For the purpose of isolating the alcohol from the precipitate, 400 microliters of Lysis buffer solution was added.

2- 20 microliters of the K Proteinase enzyme were added to sterile tubes with a capacity of 1.5 ml. That is containing the precipitate mixture after that it was mixed well with the Vortex device for 30 seconds.

3- The samples were incubated in the incubator at 60 degrees Celsius for 20 minutes.

4- 200 microliters of buffer binding solution were added to each of the sample , then they were mixed well with the vortex device for 10 minutes.

5- After that, the samples were incubated in the incubator at 60 ° C for 30 minutes.

6- 200 microliters of absolute ethyl alcohol was added and mixed well by vortex. For 15 seconds,

7- the device - the mixture was transferred to special tubes equipped with the equipment called column binding placed inside collection tubes with a capacity of 2 ml. After that, those tubes were placed in a centrifuge at a speed of rpm8000 for one minute, then the products of the decomposing cells were disposed of.

8- 500 microliters of buffer Washing 1 solution were added, after that these tubes were placed in the centrifuge at rpm 8000 for one minute, after that the precipitated solution was discarded

9- Then another 5000 microliters of buffer Washing 2 solution was added, and these tubes were also placed in Centrifuge at rpm12000 for 3 minutes, after which the precipitate was removed.

10- Those column binding tubes containing DNA were transferred to sterile tubes with a capacity of 1.5 ml, and then 50 microliters of buffer elution were added, and these tubes were quickly placed in the centrifuge. rpm8000 and for one minute to dissolve the DNA

11- After that the DNA was kept in the refrigerator until it was used for the PCR examination

Examination of the concentration and purity of the DNA

Spectrophotometer Nanodrop was used to measure the concentration and purity of the DNA by the absorbance of the DNA at Wavelength and when it is (280 - 260 nm) the absorbance ratio is (1.8) evidence that the DNA is pure and that the use of the device is as follows

1- We start the device and then we start by choosing a suitable program for selecting DNA **2-** We use blotting paper to filter the scale substrate of the device through Put 1 microliter of (ddH2O) by using a sterile pipette on the surface of the meter substrate in order to conduct the zeroing process, then clean the substrate for the purpose of measuring

3- We press the OK button to start the DNA concentration measurement process -

4-We take 1 microliter of each DNA sample that was previously extracted

5- The process of cleaning the device's measuring substrate is done again, then we examine the other sample, and so on.

6- After that, the process of determining the purity of the DNA will be done by reading the absorbance at two wavelengths, 260-280 As the purity of the nucleic acid is considered pure when the absorption rate is 8. 1

PCR examination method

A- Preparation of a multiplex polymerase chain reaction mix.

mix master PCR The polymerase chain reaction mix was prepared by using the Mix PCR kit. The Canadian company Abm, according to the company's instructions, is as follows :

1- A multiplex polymerase chain reaction mixture that was prepared in PCR tubes equipped with the kit, which contain the components of the polymerase chain reaction

2-the tubes are closed after completing the PCR preparation process and mixed carefully by the Vortex mixer for 5 seconds.

3- The tubes were transferred to the Thermocycler PCR device for the purpose of conducting thermal cycles condition Thermocycler PCR

Mix master PCR	Volume

Forward Primer (10Poml)	3ml
DNA Template	5ml
2XTaq Polymerase	25ml
Reverse Primer(10 Poml)	3ml
Nuclease free water	14ml
Total	50ml

B- Thermal cycles conditions PCR

PCR Thermocycler was used, as the device was programmed to perform a PCR test, as shown in the table

PCR step	Tempearture	Repeat cycle	Time
Initial denaturation	94	1	3 min
Denaturation	94	35	30 sec
Anneaing	55	35	30 sec
Extension	72	35	60 sec
Final extension	72	1	10 min
Hold	4	-	Forever

Electrophoresis Gel:

To read the result of the polymerase chain reaction analysis product PCR, electrophoresis was used using 1.5% agarose gel.

1- 10 microliters of PCR product were added to each well and 5 microliters of Standard DNA Ladder at 100 nitrogenous bases were taken in the first hole

2- The power supply was connected to a trough The relay is carried out through the positive and negative wires, and then an electric current of 100 volts is passed for a period of 60 minutes, and the relay is carried out towards the positive pole.

3- The gel template is lifted after the completion of the electrophoresis process to be placed in a transilluminator - UV at a wavelength of 260 nanometers To see the amplification product bundles and then photograph them using the camera installed in the device,

Method Sequencer DNA

After confirming the volume of the polymerase chain reaction products, then taking the positive samples in small tubes, and with each sample a back and front primer, and the volume of the sample sent was about 40 microliters and transferred through DHL to the Korean University ofSeoul in order to read the nucleotide sequence of each sample after sending the result via the Internet, and then reading the result through the Bioeidt program and get rid of the result with the genes of the NCBI Global Genome Bank through the use of the Blastn program and then register the genes in the NCBI Global Genome Bank and in the name of the researchers and the name of the University and compare the isolated local genetic sequences results

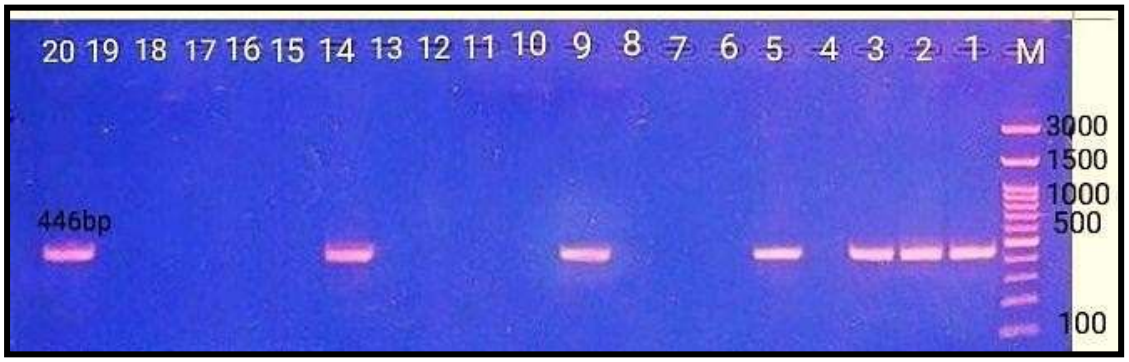
Results

Results of molecular identification Molecular:

Concentration of DNA purity: after measuring the purity and concentration of DNA of the samples taken in the study, the concentrations of the DNA ranged between 60-140 ng per microliter, and the DNA was 1.8

Polymerase chain reaction (PCR reaction chain polymerase).:

The results of electrophoresis of the DNA of hydatid cysts type on agarose gel revealed the presence of amplified COX1 gene bundles using the polymerase chain reaction (PCR) technique, with a size o**f** Pb446 nucleotides**, which** represents the diagnostic gene of *E. granulosus* . All DNA amplification processes that were extracted from theProtoscolices and germinal layer of the COX1 mitochondrial gene were successful, and the result was positive compared to the standard Ladder



Figure(1) Electrophoresis of agarose gel containing COX1 gene for PCR - technique for *E. granulosus* type DNA analysis.Sequencer

Where the ladder marker is 100-3000 M and the numbers from(1-20) represent the positive samples and the length of Pb446. Some study samples of the *Echinococcus granulosus* were recorded in the NCBI Gen Bank, and we were provided with their serial numbers, as in Table Samples registered in the Genome Bank and their serial numbers

The name of the Parasite	Serial Number	Sample number
Echinococcus . granulosus	0Q891342	1
	0Q891343	2
	0Q891344	3
	0Q891345	4
	0Q891346	5
	0Q891347	6
	0Q891348	7
	0Q891349	8
	0Q891350	9
	0Q891351	10
	0Q891352	11
	0Q891353	12

Discussion

Molecular examination

Identification Molecular PCR technique The results of the molecular examination of the samples from which the DNA was extracted, represented by the protscolices and the germinal layer, indicated the presence of diagnostic COX1 gene bundles amplified by the polymerase chain reaction (PCR) method, with a size of bp446Nucleotides, which showed that all samples are *Echinococcus granulosus*. . at a rate of 100%, and this result is consistent with many studies such as (10,11).) in humans, and that a strain specific to sheep is responsible for infecting humans in the provinces of Najaf and Qadisiyah with a rate of 33.33% and a match rate of 96% when compared with the gene bank, which bore the code KP16120701 from Africa, and another strain related to buffalo, which matched 99%, 100%, which bore the code M84663.1 from Turkey, which is also registered in the Data Bank, and the results of the study also agree with(**12**) in Al-Qadisiyah Governorate and**(13**). The current study also agrees with (**14**), in Italy, in which it was found The percentage of 87.5% infected with the parasite *E. granulosus*. It also agrees with what was confirmed in Iran by(**15**)that humans are more infected with *theE. granulosus* parasite, and the result is also consistent with what was indicated in Iran by many researchers (**9,16**), and in **(17)** and in). Brazil (**18**) . The results of the current study differed with the researcher(18) in his study that he conducted in Iraq in Sulaymaniyah Governorate, with a rate of 100% in terms of the type of parasite that causes hydatid cysts in humans. The results of our study also differed with another study conducted in the city of Baghdad, in which(**19)** confirmed for a sample of **60** patients with hydatid cysts, where it was noted that there are many different strains . The parasite is responsible for infecting humans in different proportions. Likewise, the results of the current study differed with a study conducted in Iran **by** (20), and he indicated that there are many strains that also infect humans (20-23). The reason for the differences in the results may be attributed to the size of the sample studied, the different geography of the regions, including the samples, the customs and traditions, the level of health awareness and the prevalence of hosts, in addition to the adaptation factor of these strains and according to the places of infection (24-27). This technique confirms the closeness of the diagnosis of the current study samples with the global samples, as well as the identification of strains of the nitrogenous bases of the COX1 gene of *Echinococcus granulosus* samples, as this technique is one of the accurate diagnostic methods that can be used in biological and genetic studies after comparing them with local and international studies (28-30). This rapprochement

may be attributed to the fact that Iraq is close to its borders with Turkey and Iran, in addition to the trade exchange of food and pets such as dogs

Conclusions :

The results of the current study differed with the researcher in his study that he conducted in Iraq in Sulaymaniyah Governorate, with a rate of 100% in terms of the type of parasite that causes hydatid cysts in humans. The results of our study also differed with another study conducted in the city of Baghdad, in which confirmed for a sample of 60 patients with hydatid cysts, where it was noted that there are many different strains.

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