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Molecular Characterizations of Cystic *Echinococcus* from Camels (*Camelus dromedarius*) in Al-Diwaniyah Province / Iraq

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Abstract :

The study included 25 samples of hydatid cysts from one humped Camel (*Camelus dromedarius*), which were collected by examining 632 slaughtered animals from slaughterhouses in Al-Diwaniyah province / Iraq during the period between the first of September 2020 to the end of February 2021. The aim of study to investigating the molecular characterization of hydatid cysts isolates from one humped camel (*Camelus dromedarius)* and common genotypes (strains) of the *Echinococcus granulosus* that infect Iraqi camels in Al-Diwaniyah province /middle of Iraq by using the polymerase chain reaction (PCR) technique .

The results of the technique of polymerase chain reaction (PCR) showed the success of the DNA amplification processes extracted from the protoscolesis of 25 hydatid cysts samples, as the results were positive in all samples that were used for this purpose at a molecular weight of 450 bp.

The study of the sequence of the Cytochrome c oxidase subunit 1 cox1 gene (Cox1 gene) and the phylogenetic tree analysis and comparison with the gene bank (NCBI-BLAST) to determine the common strains of camels in Al-Diwaniyah province showed presence the G3 (buffalo strain) and G1 (sheep strain) genotypes in iraqi camels (80% and 20% respectively) , G6 (camel strain) was did not appeared in this study .

The genetic analysis of the (COX1) gene indicated that the largest match of local isolates in Iraqi camels was with Iranian isolates of the same type, with a genetic match ratio ranging between 99.14% -100 % when the total genetic variation (0.010-040). Isolates were registered in Gen bank and serial numbers were given for the study, which represents the G3 strain of buffalo and G1 strain of sheep registered in this study.

Key words: *Echinococcus granulosus*, *Camelus dromedaries*, PCR, Al-Diwaniyah province**.**

1- Introduction:

Hydatid cysts disease (Cystic Echinococcus) is a zoonotic parasitic disease o that represents the larval stage of the groups of tapeworm species *Echinococcus granulosus sensu lato* and it is spread all over the world due to the life cycle of this parasite that includes dogs and carnivores as the final host of the mature form of the parasite. Its intermediate host develops into a larval form or a hydatid cyst (1), which are occasional intermediate hosts that develop echinococcosis when swallowing tapeworm eggs that are thrown into the feces of humans by a final host. And a hydatid cysts are one of the serious diseases that affect camels which act as important intermediate hosts for the *Echinococcus granulosus*, where the prevalence of *E. granulosus sensu rict, E. intermedius, E. artleppi*(2,3)

Molecular studies indicated the presence of 10 distinct genotypes of *Echinococcus granulosus* (4), and these genotypes or strains were called (G) which are G1 - G10, it different in epidemiology, pathogenicity , infection, control, appearance, and host types. The mean, geographic distribution, adult morphology, larvae, and other traits (5). The genotypes of *E.granulosus* were determined on the basis of molecular genetic analysis using DNA sequencing, phenotype, and gene sequences. These strains are *E.granulosus sensus tricto*. Genotypes: G3-G1(sheep strain),*Echinococcus equinus* (G4) and *Echinococcus ortleppi*, G5 and *Echinococcus canadensis*, which includes the G10-G6 strains, as G6 is the camel strain, G7 is the pig , G8 and G10 are the two deer strains (6). Polish patients are currently classified under the G7 strain (7).

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The G6 strain represents the camel strain, and this strain was discovered mainly in the Middle East, Iran, China, Africa and Argentina, and the final host is dogs, while camels, goats and cattle are intermediate hosts. This strain infects humans and causes echinococcosis and is widely endemic in North Africa and the Middle East (8).

The infection of Iraqi camels with hydatid cysts has major effects on an important aspect of livestock, and the high percentage of camels infected with hydatid cysts confirms that the camels as an important intermediate host in the life cycle of the *E.granulosus* and it is contribution in spreading of infection to both humans and other animals (9).

Many molecular studies have been conducted to determine the genotypes of the *E.granulosus* that infects camels in many countries, including China, Iran and Egypt, and the data clearly indicate that the camel strain , the G6 genotype as well as sheep strain G1 are the two common strains that were obtained in the north Xinjiang, China (10).

In Iran,(11,12,13)they carried out a many studies to reveal the molecular characteristics of *E.granulosus* from the hydatid cysts isolated from camels in the some provinces, and the results obtained indicated that the common genotypes of *E.granulasus* in Iran are the G1, G3 ,G5and G6.

In Egypt, the results showed the presence of the genotype G6 in 26 out of 28 hydatid cysts of camels (14), the genotype G1 in only one cyst and G5 in one cyst as well.

In Iraq, molecular studies on camels are very few and rare, and from an epidemiological point of view, this disease was considered to be hyper endemic. The reason for this is the large numbers of stray dogs infected with echinococcosis adult worms, as they shed eggs containing six-pronged embryos that infect the intermediate host (15).

In a study carried out by(16) in Al-Qadisiyah province that aimed to distinguish the genetic differences of isolates of the *echinococcus granulosus* in humans and animals, where 17 cysts samples of camels were collected from the Al-Diwaniyah city slaughterhouse and 5 samples of them were chosen for molecular analysis where three strains were found, which is the G1 sheep strain (40%),G3 buffalo strain (48%) and G6 camel strain (12%) .

Recently(3), she conducted a study on hydatid cyst disease in slaughtered animals, including camels in the Middle Euphrates provinces of Iraq ,the results of the genetic tree analysis of two isolates taken from the liver of infected local camels indicated that the G1 and G5 cattle strain may be responsible for camel infected in the Middle Euphrates provinces as it was similar to the genotypes of the global isolates registered in a Gene bank.

2- Methods

Hydatid cysts collection

In this study, 25 samples of hydatid cysts were used collected from the livers and lungs of camels, which were collected during the study period between the first of September 2020 - the end of February 2021. These samples were obtained from the slaughterhouses in the Al-Diwaniyah province. cysts was transferred to the molecular researches laboratory in the Department of biology / College of Education for pure sciences / University of Al-Qadisiyah, where each cyst was examined and prepared for subsequent operations.

Isolation of protoscolices and germinal layer

(17) method was used, where the outer surface of the infected organ and the cysts in it were sterilized using 70% ethanol alcohol to prevent contamination, then 5 mm syringes were used for the purpose of perforating the cyst and then withdrawing the sand fluid from it, as approximately 95% of it was withdrawn and placed inside test tubes of the size 10 ml, and one drop was taken from it and placed on a glass slide, then the cover of the slide was placed and examined under a light microscope with a force of 4X and 10X, and the presence or absence of the protoscolices was observed in it, and then a circumferential incision was made in the outer fibrous envelope of the cyst using a scalpel and forceps, and then the fluid containing the protoscolices was withdrawn. after that By a 5 ml syringe, the germinal layer by forceps was withdrawn and placed inside the preservation bottles and then in the

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next step based on(18). The tubes were placed inside the centrifuge at a 3000 rpm for 15 minutes and the remaining liquid was poured at the end of time and when a layer of protoscolices was noticed at the bottom of the tube in the event that the cyst was fertile, the precipitate was washed with the addition of 10 ml of normal physiological saline solution. Saline and the same process was repeated at the same speed and for 3-5 times in order to the purification of protoscolices.
The protoscolices were kept with 70% of ethanol alcohol until the DNA extraction process was performed (19).

Primers

The technique of polymerase chain reaction (PCR) was used to detect the mitochondrial gene Cytochrome c oxidase subunit 1 (cox1) gene in *E.granulasu*s. In our study, the purpose of this study is to uncover the genotypes of the *E.granulasus*causing camel hydatidosis, and by relying on(20)and using two amplification primers. At a molecular weight of 450bp, they are:
TTTTTTGGGCATCCTGAGGTTTAT Sequence 5'-3 'Forward
TAAAGAAAGAACATAATGAAAATG Sequence 5'-3 'Reverse
The process of determining the genotype took place in several steps as following :

DNA extraction

Extraction DNA from cyst samples, whether protoscolicesor generative layer, using the g SYAN DNA Extraction Kit from Geneaid Taiwan, following the manufacturer's instructions with the following steps:
200 µl of hydatid cyst fluid was transferred to a sterile 1.5 ml fine tube and placed in a centrifuge at 10,000 rpm for 1 minute. The supernatants were discarded, 200 µl of GST solution and 20 µl of proteinase K were added and mixed by vortex. Then they were incubated at 60 ° C for 15 minutes, after which 200 µl of GSB solution was added to each tube and mixed vigorously. All tubes were incubated at 60 for 10 minutes, and were turned over every 3 minutes during incubation periods. 200µl of absolute ethanol was added to the lysate and immediately mixed by vigorously shaking. Then 200µl of absolute ethanol alcohol were added to the mixture and mixed directly and vigorously with a mixer for 10 seconds. DNA purification filters called GS Column were placed in the 2 ml Collection Tube, then all the mixture, including the insoluble components, was transferred to the GS Column, and placed in a centrifuge at 10,000 rpm for five minutes to get rid of the degraded cell products .Then the collector tubes and the collected liquid were discarded and the GS Column was placed in new 2 ml collecting tubes.

400µl of the W1 buffer wash solution was added to the GS Column filters and then placed in a centrifuge at 10,000 rpm for 30 seconds to wash the DNA, after which the precipitate was discarded. 600µl of Wash Buffer (absolute ethanol) washing solution was added to each filter, then placed in a centrifuge at 10,000 rpm for 30 seconds as well. The sediment was removed and the filters returned to the collecting tubes. All tubes were placed in a centrifuge again for 3 minutes at 10,000 rpm to dry the filters. Then the dried DNA filters containing the DNA were transferred to new, sterile Eppendorf tubes, and 50µl of Pre-heated Elution Buffer were added to the medium of the DNA filters.

The tubes were left for 5 minutes to ensure that the dissolving solution was absorbed into the remaining filter components, and then placed in a centrifuge at 10,000 rpm for 30 seconds, to filter and isolate the purified DNA. Then they were stored in a deep freezer at -20 ° C until the PCR process.

Genomic DNA examination

DNA extracted from samples taken from the liver and lung of camels was detected through the use of a Nanodrop spectrophotometer (THERMO. USA) for detection and measurement of the concentration of (DNA and RNA) where DNA is detected by determining the concentration of DNA(ng\µlDNA) and measure the purity of DNA by reading the absorbance at a wavelength of 260-280nm.

Prepare the PCR master mix

The PCR reaction mixture was prepared using the AccuPower® PCR Master Mix kit equipped by the

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Korean company Bioneer and according to the company's instructions and according to the sizes and as follows:

DNA template 5µL; Forward primer 10pmol 1.5µL; Reverse primer 10pmol 1.5µL; PCR water 12µL and total volume 20µL

After that, the components of the PCR reaction mixture mentioned above were put into 0.2ml opaque tubes of the PCR machine, then all tubes were transferred to a vortex centrifuge (Exispin) at a speed of 3000rpm for three minutes and then placed in the PCR Thermocycler.

PCR Thermocycler Conditions

checked using the thermocycler. The device has been programmed: Initial Denaturation 1 Cycle in 95c for 5Min; Denaturation 30 Cycles in 95c for 5sec. ; Annealing 30 Cycles in 58c for 30sec. ; Extension30 Cycles in 72c for 3min; Final Extension 1 Cycle in 72C for 10min; Hold Unlimited Cycles in 4c Forever.

DNA sequencing and genetic tree analysis method

The DNA sequencing method for *Echinococcus granulosus*, diagnosed with a PCR examination, was performed by performing the genetic tree analysis of the cox1 gene of local isolates of the *Echinococcus granulosus* in camels to determine the genetic relationship between the present strains and the strains in other countries, especially the neighboring countries, using the calculation of development distances that were done Measured using odte hood Likeli it Maximum Compos using the UPGMAtree tool using(MEGA software version 6.0).

After performing the PCR reaction, the product of the PCR reaction was sent to Macrogen in South Korea for DNA sequencing using the AB DNA sequencing system and comparing it with a data base of NCBI (National Center for Biotechnology Information, Bethesda, MD, USA).

3-Results

DNA extraction results

The purity of the DNA was extracted from 25 samples taken from hydrated cysts of the liver and lungs of camels. The results showed that the purity of the DNA was 289.6µl / ng at the level of significance 0.05, and the mean ± standard error of it was l / ng71.46 ± 15.82.

Results of PCR for coxl gene

The results of the PCR technique showed the success of all DNA amplification processes extracted for the mitochondrial Cytochrome c oxidase subunit 1 (cox1) gene, as the results were positive for all samples, as shown in Figures (1, 2) and Table (1) where the results of DNA amplification were positive for samples from 1-25 samples Cysts after electrophoresis on agarose gel, where the presence of the diagnostic gene (COX1 genes) of the *Echinococcus granulomatous* parasite at a molecular weight of 450 bp.

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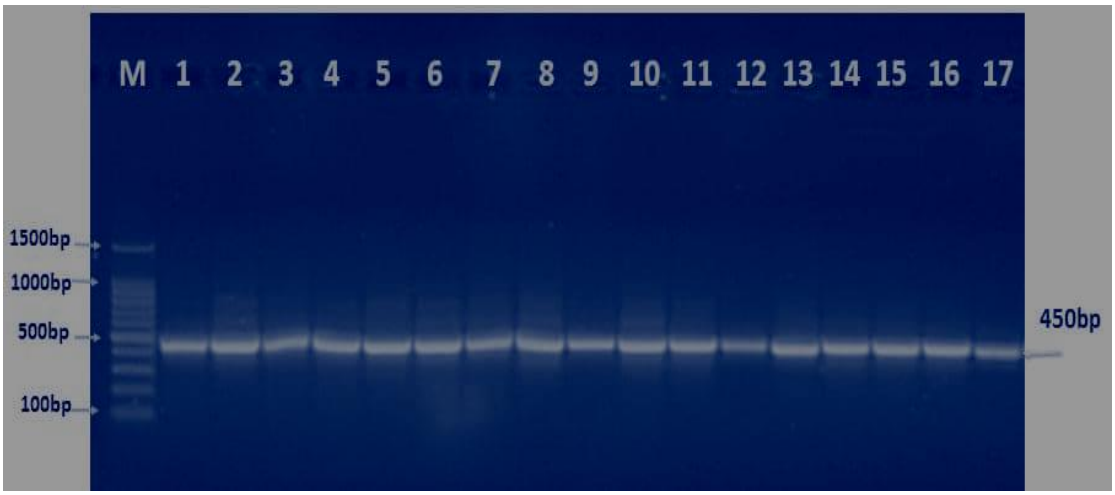

Figure (1) represents the electrophoresis on agarose gel containing the results of the PCR examination for the gene (COX1gene) for the diagnosis of the *E.granulasu*. from the hydatid cyst samples from camels, representing M: Marker ladder2000 -100bp, and numbers (1-17) representing positive samples for testing at a molecular weight of 450 bp.

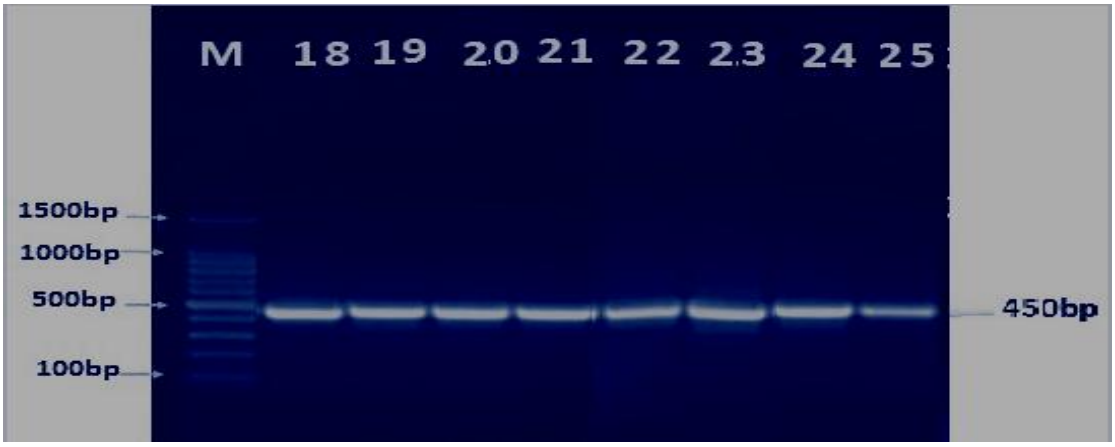

Figure (2) represents the electrophoresis on agarose gel containing the results of the PCR examination for the gene (COX1gene) for the diagnosis of the *E.granulasu*. from the hydatid cyst samples from camels, representing M: Marker ladder2000 -100bp, and numbers (18-25) representing positive samples for testing at a molecular weight of 450 bp.

phylogenetic tree analysis

During the study 25 positive randomly samples from the 192 sample group were analyzed for the DNA sequencing process. The results of the genetic tree diagram analysis of studied camel isolates based on the partial sequence of the mitochondrial gene COX1 gene of the local isolates of the *Echinococcus granulosus* in camels to determine the genetic relationship between the local strains and strains in other countries, especially Contiguousness using the computation of development distances measured using odte hoodLikeli it Maximum Compos with the UPGMAtree tool using MEGA version 6.0. The sheep strainG1genotypes and buffalo strain G3, are responsible for camel hydatidosis infected in Iraq, and the G3 genotype is the most common and widespread in camel diseases in the province (20/25 and 5/25, respectively).

Table (1) shows the identical sequence match in NCBI-BLAST between native *E. granulosus* isolates from camels with isolates of *E. granulosus* related strains recorded in NCBI-BLAST.

Isolate NO.	Gen bank Accession	Homology sequence Identity in NCBI-BLAST

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	no.	**Identical genotype**	Gen bank Accession no.	**Identity**
E.granulosus Camel No.1	MW740181	G3	MW421883.1	99.34%
E.granulosus Camel No.2	MW740182	G3	MW421883.1	100%
E.granulosus Camel No.3	MW740183	G3	MW421883.1	99.64%
E.granulosus Camel No.4	MW740184	G3	MW421883.1	99.73%
E.granulosus Camel No.5	MW740185	G1	MN732819.1	99.44%
E.granulosus Camel No.6	MW740186	G3	MW421883.1	99.34%
E.granulosus Camel No.7	MW740187	G3	MW421883.1	99.14%
E.granulosus Camel No.8	MW740188	G1	MN732819.1	100%
E.granulosus Camel No.9	MW740189	G3	MW421883.1	99.66%
E.granulosus Camel No.10	MW740190	G3	MW421883.2	100%
E.granulosusCamel No.11	MW740191	G3	MW421883.3	99.34%
E.granulosusCamel No.12	MW740192	G3	MW421883.4	99.34%
E.granulosusCamel No.13	MW740193	G3	MW421883.5	100%
E.granulosusCamel No.14	MW740194	G3	MW421883.6	99.55%
E.granulosusCamel No.15	MW740195	G3	MW421883.7	99.66%
E.granulosusCamel No.16	MW740196	G3	MW421883.8	100%
E.granulosusCamel No.17	MW740197	G1	MN732819.1	99.34%
E.granulosusCamel No.18	MW740198	G3	MW421883.1	100%
E.granulosusCamel No.19	MW740199	G3	MW421883.1	99.35%
E.granulosusCamel No.20	MW740200	G1	MN732819.1	99.14%
E.granulosusCamel No.21	MW740201	G3	MW421883.1	99.53%
E.granulosusCamel No.22	MW740202	G1	MN732819.1	99.34%
E.granulosusCamel No.23	MW740203	G3	MW421883.1	99.22%
E.granulosusCamel No.24	MW740204	G3	MW421883.1	99.33%
E.granulosusCamel No.25	MW740205	G3	MW421883.1	99.34%

Genetic tree diagram analysis of studied camels isolates showed that the local isolates of *E. granulosus* with accession numbers (MW740181, MW740182, MW740183, MW740184, MW740186, MW74018719, MW740189, MW740140, MW740187, MW740189, MW740140, M19740, MW740190, MW740189, MW740140, M740196, MW740198, MW740199, MW740201, MW740203, MW740204 and MW740205) the closest genetic similarity to the isolates of *Echinococcus granulosus* of the Iranian G3 strain with the serial number (MN421883.1) registered in the gene bank (NCBI-BLAST) ranged between (99.14% - 100%)with a ratio of approximately While the local specimens numbered (MW740185, MW740188, MW740197, MW740200, MW740202) were also close to the Iranian isolation belonging to the G1 strain with a accession number (MN732819.1) registered in the gene bank (NCBI-BLAST) with a ratio of convergence between 99.14-100 % At total genetic variation (0.010-040).

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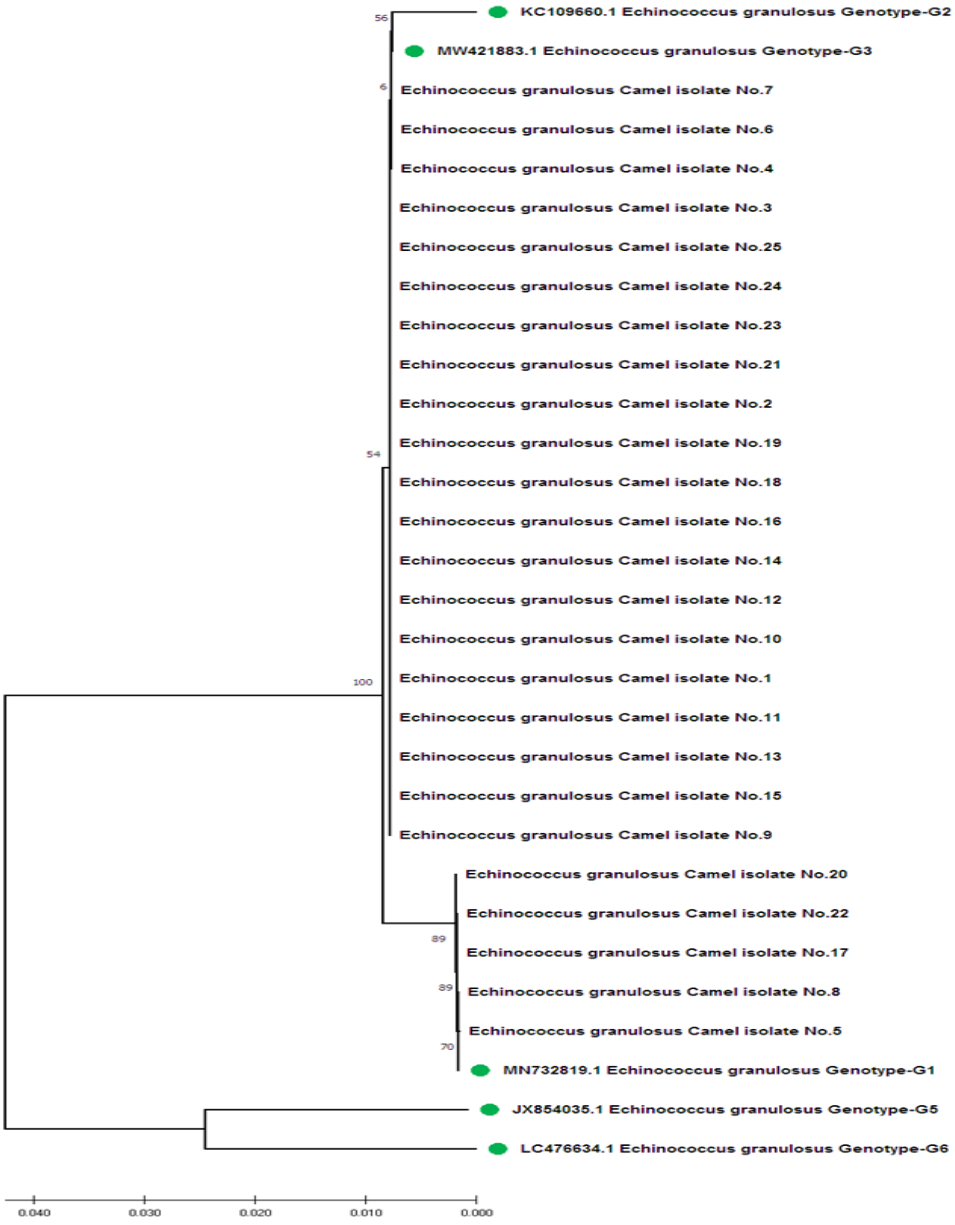

Figure (3) represents the genetic tree analysis of camel samples based on the partial sequence of the mitochondrial gene COX1 gene that was used to locate the *E.granulosus*.

Relationship of detected genotypes in this study with some genotypes in Iraq and relative countries in animals

The genetic analysis of the (Cox1) gene indicated that the greatest match for local isolates from camels in the current study was with Iranian isolates. The genetic tree of camel isolates was written using the evolution distance, which was measured using the aforementioned program, as the local camel isolates were compared with some other local isolates in (NCBI-Blast), so all isolates (25-1) showed a closer match with the Iranian isolation (MH397257. 1) upon total gene heterogeneity (0.0150-0.0050)

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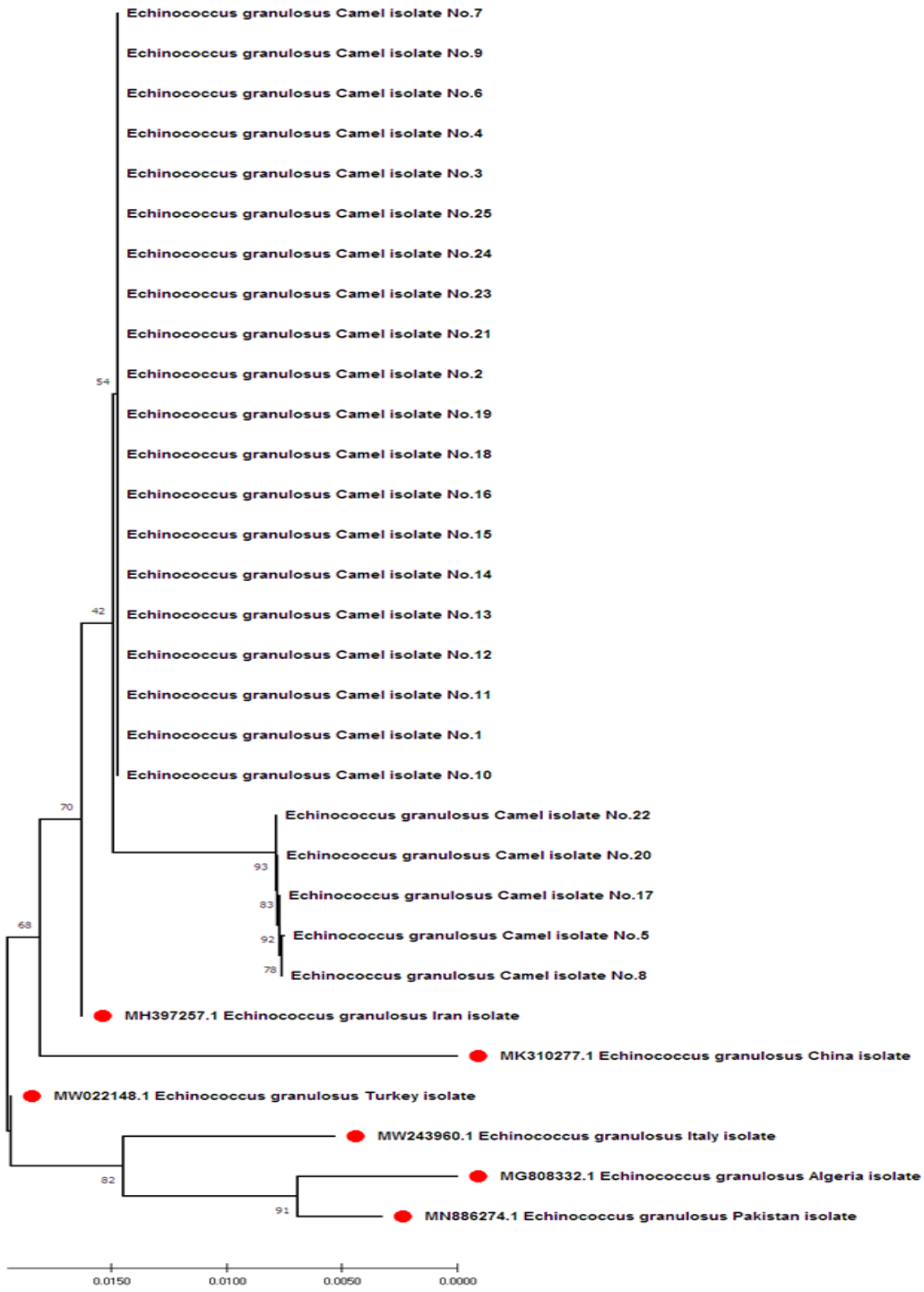

Figure (4) Analysis of the genetic tree of camel samples based on the partial sequence of the mitochondrial gene (COX1 gene) that was used to determine the strains of the current study with other local strains and some strains from nearby countries.

Results of multiple sequence alignment analysis

Multiple alignment analysis of the molecular sequence of the mitochondrial gene (cox1) in local *E. granulosus* isolates from camels, as shown in Fig.5, showed its conformity with the genotypes of the parasite registered in the International Gene bank (NCBI-Blast) using the Multiple alignment analysis tads. (MEGA 6.0) where the analysis showed regions of similarity (columns marked *) and genetic variation (substitution mutation) identified with different colors from each column in the mitochondrial gene nucleotide sequence (Cox1).

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Figure (5) shows the multiple-sequence alignment of the mitochondrial gene partial sequence (COX1) in local *E.granulosus* isolates from camels, and their correspondence with the genotypes of the parasite recorded in (NCBI-Blast).

4-Discussion

The results of the current study showed that the purity of the DNA in the samples extracted from camels , was of a unit of 260OD / 280OD, so the range was 289.6, with an arithmetic mean (±) standard error of71.46 ± 15.82. The current study differed with(3) in the central Euphrates provinces in Iraq, as it was the purity of the DNA in camels is 0.89-2.07. The difference in purity may be due to the sample size and the nature of the extraction.

Distribution of PCR for (COX1)gene

The results of the PCR technique showed the success of the DNA amplification processes extracted from the protoscolices and germinal layer of all samples, as the results were positive in all 25samples for the diagnostic gene (COX1)gene for *E.granulosus* at a molecular weight 450 base pairs (bp) after amplifying them with the technique of polymerase chain reaction (PCR) and conducting electrophoresis on agarose gel, and this is in agreement with many studies in Iraq,(21,22)and also agreed with the study (23) It was conducted in Al-Qadisiyah province and also similar to the study of(14) in Egypt, where all PCR amplification operations of samples extracted from the germinal layer of each (COX1 gene) were shown to be successful at a molecular weight of 450bp.

Distribution of DNA sequencer technique and phylogenetic tree analysis *of E.granulosus* genotypes

The results of the current study showed that the sheep strain (G1) and the buffalo strain (G3) were responsible for infecting camels in the Al- Diwaniyah province, the buffalo strain G3, appeared in 20 samples with a percentage 80%where out of 25 samples the sheep strain G1 appeared in only 5 samples As for the(20%).. The current study also agreed with the study of (3) in Iraq, where the results showed that the sheep strain G1 is responsible for the infections of camels and differed with it in the emergence of the G5 cattle strain, as it is also responsible for camel infections in the central Euphrates provinces.(24,25,26) who recorded the presence of the G1 sheep strain and the G3 buffalo strain in

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camel infections in Iran and also agreed with the study of (27) who found that the G1 sheep strain is one of the prominent strains in camel infections in Iran and also agreed With my studies (28,8), as they indicated the prevalence of infection with the G1 sheep strain in camels in Libya and Kenya, respectively.

It also agree somewhat with the study(23) in Al-Qadisiyah province, which showed that the G3 buffalo strain is responsible for camel infections in the province, and also found that the G6 camel strain is responsible for some camel infections in the same study, and with study of (16) in Al-Qadisiyah province as well They found that 20% of camel infections are caused by the G1 sheep strain, 40% of the infections are caused by the G3 buffalo strain, and 40% by the G6 camel strain. Also partly in agreement with the study by (12) in Iran, where the study found that the sheep strainG1 percentage was 28.6%, the G3 strain was 28.6%, the G6 camel strain was 35.7%, and only one sample carried the genotype of the cattle strain G5.

While the current study differed with the study of (13) in Iran, where they found that there are three genotypes responsible for camel injury, which is the G6 camel strain, which is the most representative genetic makeup of camels, followed by the G1 genotype (sheep strain), and then the genotype G5 (Bovine strain) by a small percentage.

The current study also differed with the (14) in Egypt, where 26 samples were out of 28 samples taken from camels, the strain in which they appeared was the G6 camel strain, while the G1 sheep strain appeared in only one cyst of camel samples, as for the last sample of camels, the G5 cattle strain appeared in it. In agreement with the study (29,30) in Iran, where the results showed that most cases of echinococcosis were caused by the G1 sheep strain. It also differed with the study of (21). As they recorded that the G5 buffalo strain is the most common in camels infections in Sudan, and it also differed with the studies(25,31)

Those who indicated that the most common strain in camel infections in Iran is the G6 camel strain, and also in some areas there are infections caused by the G3 buffalo strain which are common. Our current study also differs from (32,33) that was conducted In Africa, they found that the G6 camel strain is common in some African countries such as Algeria, Sudan and Mauritania.

The reason for the differences that occurred between the different studies in identifying the strains causing infection in camels is the difference in the size of the studied samples, as well as the difference in the studied geographical areas, as well as the adaptation of the strains to those areas, as well as the feeding and grazing in those areas, as well as the customs and traditions of the inhabitants of those areas, and this is consistent with (34).The results also showed that there is a similarity between the isolates of the current study with Iranian isolates more than other isolates, and this is due to the fact that Iraq shares large borders with Iran, and the reason may also be due to the wide trade exchange of fruits and vegetables and the trade of animals such as dogs from Iran, and perhaps due to the lack of studies on this parasite is found in neighboring countries such as Syria, Saudi Arabia, Kuwait and Jordan, with the exception of Turkey, in which many studies have been published in this regard.

5-Conclusions

It is concluded through the study that there are two common strains in camels infection with *Eechinococcus granulous* in Al-Diwaniyah province are G1 sheep strain, which is the most common strain followed by the G3 cattle strain.

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