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Genetic characterization of *Echinococcus granulosus* in sheep and cattle: Evidence of G1 strain predominance in Kurdistan, Iraq

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ABSTRACT

Echinococcus granulosus, a dog tapeworm, is the source of the silent helminthic illness known as cystic echinococcosis. This investigation included molecular genotyping of cysts obtained from the different organs of infected sheep and cattle in the abattoir. A total of twenty-eight echinococcal cysts were isolated from liver and lungs of fifteen sheeps and thirteen cattles. Collected samples were analyzed using the mitochondrial *cox1* gene. Gel electrophoresis was used to verify the produced amplicons. The results showed that a fragment of 446 bps was revealed. Four amplicons are present in these samples that were selected for sequencing, genotyping, and phylogenetic analysis, which was constructed by comparing the obtained sequences (MW599304 and MW599308 for sheep and MW599306 and MW599307 for cattle) with those recorded in the NCBI-BLAST. The nucleotide sequence alignment with the recorded nucleotide in the NCBI indicated that all samples of the sheep strain belong to G1. According to the findings of this investigation, the G1 strain of sheep predominated, and this strain generally is responsible for echinococcosis in animals and humans.

Keywords: *Echinococcus granulosus;* G1 Sheep strain; Hydatid Cysts; Molecular genotyping; Phylogenetic Analysis.

1. Introduction

One of the silent helminthic zoonotic illnesses, the *Echinococcus* species, which causes cystic echinococcosis, the dog tapeworm (family Taeniidae). It is found in most parts of the world as an endemic disease (Romig et al., 2015; Karamian et al., 2017). In Europe, South America, China, Australia, Northern Africa, the Middle East, and Central Asia, the cystic echinococcosis is prevalent impacting people, wild and domestic animals (Deplazes et al., 2017; Schmidt and Roberts, 2000). The life cycle of this parasite has two hosts, the definitive host, which involves canids (dogs, wolves, etc.), and the intermediate host, involving most herbivores and humans (Romig et al., 2017). The illness is characterized by prolonged growth in the body of the intermediate hosts (Mohamed et al., 2017). The mature worm inhabits canids' tiny intestines, mainly wolves and dogs (Karamian et al., 2017; Mulinge et al., 2018). At the same time, the parasite's larval form grows in several bodily organs, primarily the liver and lungs. Eggs produced by adult worms are excreted with dogs' stools to pollute water, crops, and vegetables (Otero-Abad and Torgerson, 2013). Intermediate hosts (such as cattle, sheep, goats, pigs, etc.) acquire infection by grazing on vegetation and drinking the water contaminated with the parasite, eggs excreted with the final hosts' feces (Deplazes et al., 2017). The larvae (oncosphere) emerge from the eggs in the tiny intestine and pass through the gut wall and grow into cystic structures in various "body organs" (Heath et al.,

2006). The intermediate host's echinococcal cysts can develop in various organs; the two most common sites of infection are the liver and lungs, but in addition, the infections can occur in spleens, kidneys, heart, muscles, bones, brain, eyes, and other organs (Geramizadeh, 2013). Cystic echinococcosis is regarded as a neglected illness, and it remains a public health concern because the only choice is surgical cyst removal, and the recurrence of the disease is still high depending on the operation's accuracy and techniques used (Hama et al., 2012).

Modern and accurate techniques for identifying *E. granulosus* strains are molecular ones which depend on the mitochondrial genes for ITS1, COX1, and NAD1 being analyzed using DNA (Eckert et al., 2001; Faruk et al., 2017). The parasite has ten genotypes (G1-G10) characterized globally and isolated from numerous intermediate hosts, such as sheep, horses, goats, pigs, camels, cattle, and cervids (Sánchez et al., 2010; Hu et al., 2015; Ebrahimipour et al., 2017; Hodžić et al., 2018). Molecular studies based on mitochondrial genes have identified a number of genotypes/species of *E. granulosus s.l.* complex. This complex consists of genotypes G1, G3-G8, G10 and E. felidis (which have been assigned as distinct species). *E. granulosus sensu stricto* (s.s.; comprising genotypes G1 and G3; *E. equinus* (G4) and *E. ortleppi* (G5) (Thompson and McManus, 2002).

The species status of genotypes G6-G10 has been controversial. While a study by Yanagida et al. (2017), suggested that there are sharing of some nuclear alleles between the genotypes G6/G7 and G8-G10. Later Laurimäe et al. (2018) suggested that G6/G7 and G8-G10 are two distinct species. Many studies on the molecular characterization of *E. granulosus* reported variable strains globally (Ebrahimipour et al., 2017; Laatamna et al., 2018; Mousa et al., 2020; Kim et al., 2020; Šoba et al., 2020; Mahmood et al., 2022). In Iraq, hydatid cysts are an endemic illness in animals, and much research has been conducted on the molecular characterization of these parasites (Fadhil and A'aiz, 2016; Hassan et al., 2016; Alsaady and Al-Quzweeni, 2019; Mahdi et al., 2020). In the Kurdistan region of Iraq, some studies were performed on the molecular characterization of this parasite in domestic animals in the Duhok province (Ahmed et al., 2013). Erbil province (Hassan et al., 2017; Abdulla et al., 2020) and Sulaimani province (Hama et al., 2012; Hama et al., 2015; Hama et al., 2018). The purpose of the current work is to identify *E. granulosus* strains by characterizing cystic echinococcosis (CE) at the molecular level that are infecting sheep and cattle in Zakho City, Duhok Governorate, Kurdistan Region, Iraq.

2. Materials and methods

Sample collection

Twenty eight echinococcal cysts were collected from liver and lungs of sheep and cattle in the Zakho abattoir from January 2020 to February 2021 (Table 1). The collected cysts were brought to the laboratory of the Biology Department, College of Science, University of Zakho, in labeled clear plastic bags, and washed by phosphate buffered saline (PBS). The cyst's germinal layer, with or without it, included protoscolices and was preserved in sterile, clearly labeled, sealed containers with 70% ethanol at a temperature of -20°C for subsequent DNA extraction (Sharbatkori et al., 2009; Latif et al., 2010)

Table1. Number and location of the collected cysts

Intermediate hosts	Cyst location		Total number
	Liver	Lungs	
Sheep	7	8	15
Cattle	6	7	13
Total	13	15	28

DNA extraction

To extract DNA from tissue using GeNet Bio's PrimePrep TM Genomic DNA Extraction Kit, complete genomic DNA was isolated from the 28 samples using 20 mg of the germinal layers or protoscolices. After that, the DNA was stored at -20°C until PCR was performed.

PCR process

In order to amplify and genotype the mitochondrial cytochrome oxidase subunit 1 (cox1) gene, the Thermocycler Amp PCR system 9700 was utilized. The amplification was conducted at the molecular laboratory of the Biology/Zakho University. One set of primers

was used for molecular studies of *E. granulosus* to amplify fragment of 446 bp in both forward and reverse directions, with sequence of 5'TTT TTT GGG CAT CCT GAG GTT TAT3' and 5'TAA AGA AAG AAC ATA ATG AAA ATG3' (Bowles et al., 1992a).

For the PCR run, a PCR reaction mixture of 20 µl was made by mixing 10 µl of master mix reaction and 2 µl of product DNA (template) extracted from the protoscolices or germinal layer of echinococcal cysts was added to the tube and mixed gently. After that, 5 µl of distal water were then added and carefully stirred. Then, 1.5 µl of each reverse and forward primer (cox1) were added and mixed gently. According to Sánchez et al. (2010), the thermal cycler was configured as follows: three minutes of preliminary denaturation at 95°C, sixty seconds of denaturation at 95°C, sixty seconds of annealing at 56°C, ninety seconds of extension at 72°C, in 35 cycles, and then three minutes of final extension at 72°C. For PCR product visualization, 5 µl of the PCR product were loaded into the individual wells slowly, and 5 µl of DNA ladder (100 - 1000 bps) was loaded in the first well, and a 1.5% agarose gel electrophoresis was performed using 1X TBE buffer at 80 V for 50 minutes, and color with RedSafe stain. The size of specific fragment identification was done using a 100 PBS ladder under a UV transilluminator and digitally photographed (Sambrook and Russell, 2001; Pezeshki et al., 2013).

Phylogenetic analysis and sequencing

The amplified 8 samples of PCR products were selected according to the sheep's cyst location (liver and lung). Cattle have excellent DNA quantity and purity. To sequence each sample, 30 µl of PCR products and 20 µl of 10 pmol primers were put into 1.5 ml tubes and shipped to Macrogen Company (Korea) for DNA sequencing and sequence analysis database. The produced DNA sequences were identified using the BLAST. By using the BioEdite program, all sequences were cleaned up and aligned.

Results

DNA was taken out of the germinal layers or protoscolices of 28 cysts that were separated from various intermediary hosts in the livers and lungs, including 15 sheep and 13 cattle. Specific primers designed for PCR amplification of the mitochondrial cytochrome subunit 1 (cox1) gene of *E. granulosus*, which has been often used to identify variants (Bowles et al., 1992a). The partial mt*cox*1 gene of all samples was successfully amplified, and all samples showed an amplicon size of 446 bps by using agarose gel electrophoresis, as seen in Fig. 1 and 2.

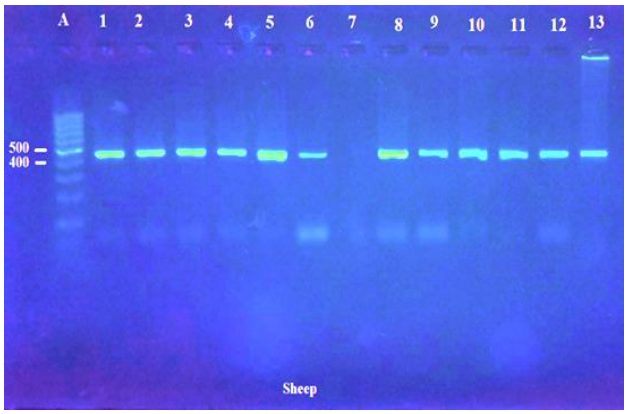

Fig. 1. Electrophoresis of PCR products for the *cox1* gene: A = ladder (100-1000 bp); lanes 1, 2, 5, 11, and 13: CE isolated from fertile sheep liver, lane 6: CE from sterile sheep liver. Lanes 3, 4, 7, 10, and 12: CE isolated from fertile sheep lungs, lanes 8 and 9: CE from sterile sheep lungs.

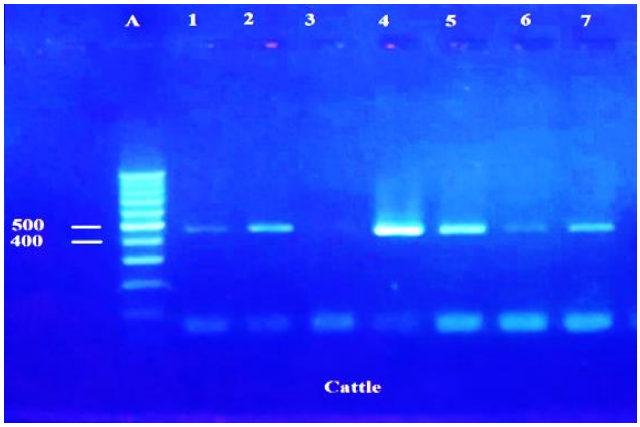

Fig. 2. Electrophoresis of PCR products for the *cox 1* gene A = ladder (1000 bp). Lanes 1 and 2 from cattle lungs are fertile CE; lanes 3, 4, and 5: CE from cattle sterile lung; lane 6: CE from cattle liver fertile, lane 7: CE from cattle liver sterile.

The sequences analysis of *E. granulosus*

Out of 28 PCR products, only eight products were sent for DNA sequencing. Four sequences have been recorded in the NCBI database

(GenBank) under the accession numbers MW599304 and MW599308 from sheep and MW599306 and MW599307 from cattle. The acquired accession numbers and their sequences were contrasted with the majority of *E. granulosus's* related sequences, which were previously published in GenBank under the accession numbers: Kyrgyzstan (MN787525, MN787547, MN787548, and MN787549), Iran (MN807920 and MN807917), Ukraine (MT380912), and Turkey (MN328343), as shown in table 2. The results of this investigation demonstrate that *E. granulosus* in cattle and sheep is similar to the sheep strain G1 (*E. granulosus* sensu stricto), based on the accession number in GenBank.

The sequences analysis of *E. granulosus* shares 99.1% - 100% homologies to the isolates from: Kyrgyzstan (MN787525, MN787547, MN787548 and MN787549), Iran (MN807920 and MN807917), Ukraine (MT380912) and Turkey (MN328343) (table 2).

The phylogenic analyses

The phylogenic analyses showed that *E. granulosus* isolates were distributed into two genetically distinct clades. The first clade includes the following sequences (MW599304, MW599306 and MW599308) and the second clade contains the well-supported monophyletic sequences MW599307 (Fig. 3).

The first clade includes sequences obtained in Kyrgyzstan, Iran and Ukraine, while the geographic distribution of the second clade was restricted to Turkey. The phylogenic tree showed that IQ-isolate MW599307 is monophyletic in the second cluster with Bootstrap 98% and forms a clade with Turkey isolate MN328343. The obtained sequences, MW599304 and MW599306, are sister taxa in one cluster. The phylogenic tree was built by comparing the obtained sequences with their accession numbers with references from GenBank sequences of *E. granulosus*, as shown in Fig. 3, using the MEGA-6 software program

Table 2. Accession numbers of isolates of *E. granulosus* from infected animals at Zakho slaughterhouses

Species	Accession No.	Host	Region	Similarity%
E. granulosus	MW599304	Sheep/liver	Iraq	---
	MN807920	Human	Iran	99.19
	MN787525	Dog	Kyrgyzstan	99.19
	MW599306	Cattle/lung	Iraq	---
	MN787549	Human	Kyrgyzstan	99.73
	MN787547	Human	Kyrgyzstan	99.73
	MW599307	Cattle/liver	Iraq	---
	MN328343	Sheep	Turkey	100
	MN807917	Human	Iran	99.73
	MW599308	Sheep/lung	Iraq	---
	MN787548	Human	Kyrgyzstan	100
	MT380912	Pig	Ukraine	100

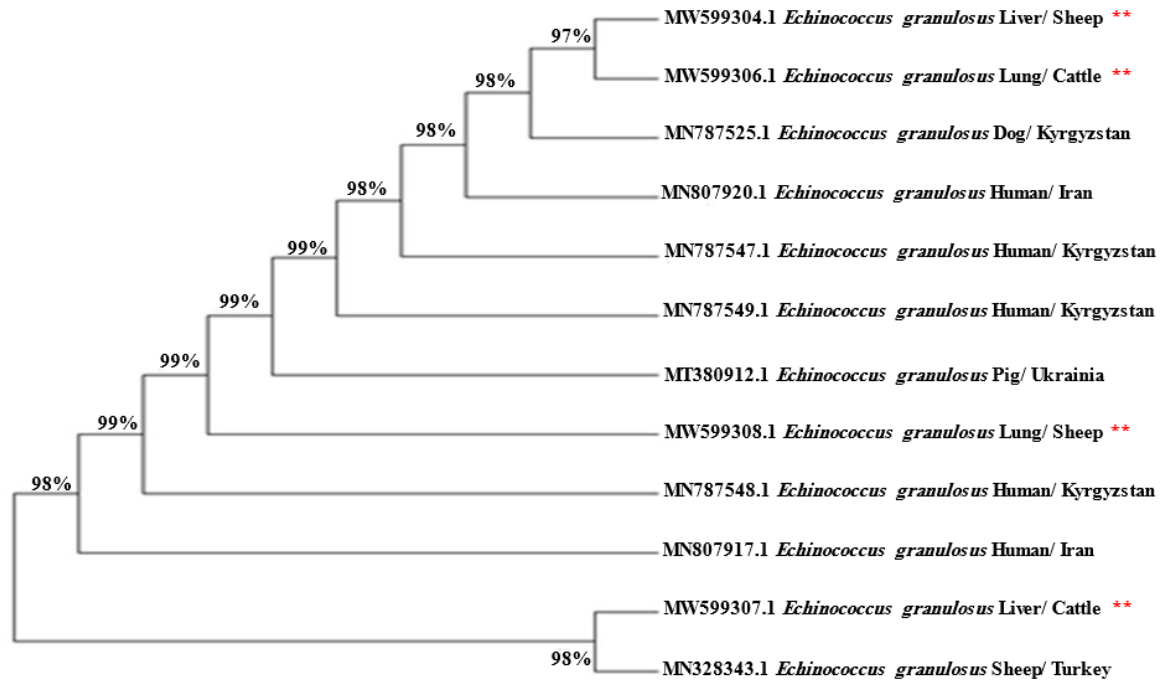

Fig. 3. Phylogenetic relationship of CE isolated from sheep and cattle slaughtered at Zakho abattoir. The phylogenic tree was created with Bootstrap and MEGAX VERSION 10.

4. Discussion

The results of this investigation, as indicated by the close relatedness to the accession numbers from GenBank, show that the strain of Echinococcus responsible for animals' infections in the Duhok governorate is the G1 sheep strain (*E. granulosus* sensu stricto), as this strain has a global distribution. The findings of this investigation are consistent with those of other studies carried out in Iraq and other nations, which demonstrate that the sheep strain's G1 genotype predominates in the Kurdistan area (Hama et al., 2012; Hama et al., 2018; Abdulla et al., 2020), in Kirkuk (Hassan et al., 2016), and Misan (Alsaady and Al-Quzweeni, 2019), in Peru (Sánchez et al., 2010), and in Egypt (Amer et al., 2015), which can infect the majority of intermediate hosts, such as people and animals like sheep, cattle, and goats. In Iraq, few studies were conducted involving molecular analysis of CE.

However, in many molecular studies, both G1 and G3 were reported (*E. granulosus* sensu stricto), such as in Romania (Piccoli et al., 2013), in Al-Qadisiyah (Fadhil and A'aiz, 2016), China (Guo et al., 2019), in Al-Diwaniyah (Mahdi et al., 2020), and in Iran (Barazesh et al., 2020). Furthermore, several studies have demonstrated that *cox1* is capable of identifying the strains and genetic variation among *Echinococcus* strains with excellent accuracy. There are striking similarities between the DNA sequences of the pmtcox1 gene, the G1 strain of sheep, the G2 strain of Tasmanian sheep, and the G3 strain of buffalo (Bowles et al. 1992b), based on Nakao et al. (2007). The genotype G1 of *E. granulosus* has the widest spectrum of hosts and is the most prevalent genotype worldwide (Craig et al., 2003). In other investigations (Obwaller et al., 2004; Hassan et al., 2017), the strains G1, G2, and G3 are all a part of a single cluster called *E. granulosus* sensu stricto. The high prevalence of sheep strain (G1) may be due to the Kurdistan region's tight trade relations with its neighbors; most butchered sheep and cattle are imported from Turkey and Iran, which are *E. granulosus* epidemic zones (Pour et al., 2011).

Conclusion

The mitochondrial cytochrome oxidase subunit 1 (cox1) gene of *E. granulosus* was amplified using a specific primer that produced a 446-PBS fragment. This study concludes that the sheep strain G1 of *E. granulosus* is the

common strain in Zakho city, which infected ruminants (sheep and cattle) since this genotype has many livestock animals serving as intermediary hosts and the Kurdistan region/ Iraq is an endemic area of *E. granulosus*.

Ethics approval

This research has been granted approval by the Zakho University Animal Ethics Committee, adhering to their guidelines and in alignment with the principles of laboratory animal use and care.

Competing interests

The authors declare no competing interests

Authors' Contribution

All the authors made substantial contributions to this article. Wijdan M.S. Mero and Sardar Hassan Arif conceived and designed the work. Araz Ramadhan Issa collected data and samples from animals, performed the molecular analysis and wrote the first draft. Wijdan M.S. Mero and Sardar Hassan Arif revised and analyzed the data and manuscript. All authors have read and agreed to the published version of the manuscript.

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