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# Evaluation of *Echinococcus granulosus* DNA extracts from protoscoles and germinal layer in sheep

Jenan M. Khalaf [1] and Ali F. Hassan[1*]
[1]Dept. of Vet Internal and Preventive Medicine / College of Veterinary Medicine/ University of Baghdad, Iraq

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***Corresponding author:**
**Email address:**
ali_alsaade30@yahoo.com

## Abstract

***Cystic echinococcosis (CE)*** caused by the metacestode of the dog tapeworm *Echinococcus spp*., is a global zoonotic infection. It is economically important and constitutes a major threat to public health in many countries. Strains characterization is essential for the establishment of a preventive and control strategy in every endemic area. This study was aimed to compare between DNA extracts from Protoscoleces and germinal layer of *E. granulosus* strain in infected sheep. Thirty, fresh fertile hydatid cysts from sheep's infected organs were collected from different abattoirs of Baghdad, Iraq. All cysts were examined by light microscope to investigate the protoscoleces viability. Protoscoleces and germinal layer were seperated and DNA was extracted. Efficiency of the DNA extract was determined by degree of its success in PCR amplification. Genomic DNA mini kit and primers forward JB3 / reverse JB4 were used to extract DNA. The results showed that DNA extract from Protoscoleces were more visible and more concentrated than the germinal layers DNA and appeared at 448bp on electrophoresis. In conclusion, the result of this study revealed that Protoscoles DNA was differed and better than germinal layer DNA.

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## Introduction

Hydatid disease is a zoonotic disease known to spread worldwide. It is caused by larval stages (metacestodes) of tape worm parasite of genus *Echinococcus granulosus* (*E. granulosus*). It is one of the most important serious parasitic diseases in the medical, veterinary sciences and with economic consequences in different regions of the world that infect different animal species (Rausch, 1995). These domestic animals include sheep, goats, cattle, swine, buffalos, horses, and camels (Bryan and Schantz, 1989). Human beings may also serve as dead-end hosts (Binhazim *et al*., 1992). *E. granulosus sensu lato* shows intraspecific variation in relation to host, specificity,

epidemiology, morphology, developmental biology, biochemistry and genetics (Thompson and McManus, 2002). Ten different genotypes, among which G1 (sheep strain) have been formerly characterized by several researchers based on genetic characterization and they were mostly depend on the homology of the sequence of the two mitochondrial genes; cytochrome C oxidase subunit 1 (CO1) and reduced nicotinamide adenine dinucleotide subunit 1 (ND1), ( McManus, 2002; Lavikainen et al., 2003; Snábel et al., 2009). *E. granulosus* complex has been divided into *E. granulosus* sensu stricto (G1–G3), *E. equinus*(G4), *E. ortleppi* (G5), *and E. canadensis*(G6–G10), according to the new molecular phylogeny of the genus *Echinococcus* (Thompson and McManus, 2002; Nakao et al., 2010; Hüttner et al., 2008). Simseka and Eroksuzb, 2009 were study the molecular characterization of cyst material, mitochondrial cytochrome oxidase subunit 1 (mt-CO1) , gene region amplifying and sequence analyses and they found that sequence corresponding to mt-CO1 gene was identical to a sequence reported for common sheep strain (G1) . This study was designed to compare between DNA extracts from protoscoles and germinal layer of E. granulosus strain that infect sheep.

## Materials & Methods

### Parasite specimens collection

Thirty fresh fertile hydatid cysts were collected from sheep's infected organs from different abattoirs in Baghdad, Iraq. The viability of protoscoleces were determined by light microscopic test. All Hydatid cysts were washed with distal water. The hydatid cysts fluid was aspirated. Protoscoloces and the germinal layers were collected and washed with sterile phosphate buffered saline (PBS), and stored in 70% (v/v) ethanol at -20 $^0$C until DNA extraction.

### DNA extraction

Samples from each individual cyst were processed as an isolate for subsequent characterization. The protoscoleces and germinal layers were rinsed several times with PBS to remove the ethanol prior to DNA extraction. DNA was extracted from protoscoleces and from the germinal layers using the DNA extraction kit according to the manufacturer's protocol. The extraction method is briefly as the fellow: Animals tissue (cyst + protoscoles) ( 30 mg) were cut and kept in 1.5 ml micro-centrifuge tube provided by micro-pestle. The tissues were grinded and homogenized by adding 200µl of GT buffer and 20µl of proteniase K. The samples were shaken vigorously and incubated at 60 $^0$C for 30 minute to lyse the tissues. Later on 200µl of GBT Buffer were added with vigorously shaking for 5 minutes. The samples were incubated at 60 $^0$C for at least 20 minutes until the lysate was clear. Absolute ethanol (200 µl) was added to the sample with continuous vigorously shaking. Samples were placed in GD Column in a 2ml collection tube and centrifuged at 14-16,000 Xg for 2 minutes. Later on, the GD Column was replaced by a new 2ml collection tube, and 400 µl of W1 buffer were added to the GD Column and centrifuged at 14-16,000 Xg for 30 seconds, these steps were repeated for 3 times and 600µl of buffer were added to the GD Column and centrifuged at 14-16,000 Xg for 30 seconds. The flow was discarded and GD Column was placed to a new 2ml collection tube. Then 100µl of pre-heated

Elution buffer were added to the center of the column matrix and kept to stand for at least 5minutes to ensure that the Elution buffer was completely absorbed. The samples were centrifuged at 14-16,000 Xg for 30 seconds to elute the purified DNA. The extracted DNAs and PCR products were loaded on separate 1 and 1.5% TBE (Tris 0.09M-Borate 0.09M-EDTA 0.02M) and 1gm agarose gel were added and kept for 5 minutes in oven and 3µl ethidium bromide was added for staining (all Biobassic / canada). Electrophoresis carried out for 45 minutes at 80 V and the bands were visualized in UV Transilluminator.

**DNA amplification**

One target sequences of the mitochondrial DNA coding for CO1 PCR-amplified by using Genomic DNA mini kit (origin Bioneer Korea). Two conserved primers, JB3 (forward)5-TTTTTTGGGCATCCTGAGGTTTAT-3 and JB4 (reverse): 5-TAA AGAAAGAACATAATGAAAATG-3 (Busi et al., 2007), were used to amplify the mtDNA region corresponding to the part of the CO1 gene Bowles et al. (1992). The amplification reactions were carried out in a PCR thermal cycler Dice (bioneer , korea); and it was stained with ethidium bromide and photographed. The PCR programs were: 5 min at 95 ◦C (initial denaturation), 35 cycles of 50 s at 94 $^0$C, 50 s at 45 $^0$C and 50 s at 72 $^0$C and finally 10 minutes at 72 $^0$C (final extension).

**Results and Discussion**

There is little published information about the genetic characterization of protoscoleses and germinal layer of the E. granulosus in sheep in Iraq. The mitochondrial CO1-PCR with JB3/JB4.5 primers were yielded a 448 bp sized product in the sample. The PCR results of these sample were give positive for *E. granulosus*. The DNA extracted from the protoscoles and some sample of germinal layer give negative result (Figure.1). DNA extracts in this study are in agreement with the results of Simsek and Eroksuz, (2009), (Genbank accession number: KC660075.1).

The genetic strains of *Echinococcus granulosus* parasites occurring in sheep and cattle in Turkey were determined previously by Vural et al. (2008) using DNA sequencing of part of the mitochondrial Cytochrome C oxidase 1 (cox1) gene. They examined a total of 112 hydatid cysts from sheep (100 isolates) derived from widely distributed sites within Turkey as well as from cattle (12 isolates) from the Turkish province of Kars. Haplotypes were identified which corresponded clearly to the previously described strain G1 in a total of 107 isolates, including 98 isolates from sheep and 9 isolates from cattle. They found that five isolates, including 2 sheep and 3 cattle, were determined to belong to the G3 genotype. The infected animals in this study originating from Baghdad regions of Iraq, had either history of previous rural life or were still living in rural areas. The sheep's strain (G1 genotype) of *E. granulosus* is the Commonly wide distributed strain around the world. It has been found to be dominant strain both in human and animals (Thampson and Mc Manus, 2001; Ahmadi and Dalimi, 2006; Varcasia et al., 2006; Bart *et al*., 2006b; Li et al., 2008). The majority of the samples were 100% identity with sheep strain G1 from protoscoles (GenBank KC660075.1). The present study has given interesting result with CO1 gene amplification (Nejad et al., 2011) and sequencing when applied to the small samples

but it is difficult for large samples, to resolve these problem further studies recommended to be done to find specific primer for each strain. Nowadays specific primer for sheep strain were designed by (Dinkel et al.,2004), which is reliable method for molecular epidemiology to be applied on the large sample size. Utuk et al., (2008), examined 179 sheep, 19 cattle, 7 goats, 1 camel, 1 dog and 1 human isolates by using RCR-RFLP of ribosomal ITS1 gene region and mitochondrial CO1 sequence analysis and determined only sheep strain (G1) in all samples. *E. granulosus* sheep's strain were also reported previously as predominant in Kurdistan/Iraq and it was mostly responsible of human hydatid disease in Kurdistan/Iraq (Hama et al .,2012) , which is in agreement with results reported from neighboring Iran and Turkey(Ergin et al.,2010). In conclusion, the result of this study approved *E. granulosus* sheep's strain in Baghdad province and supports the fact that sheep's strain is the most worldwide distribution.

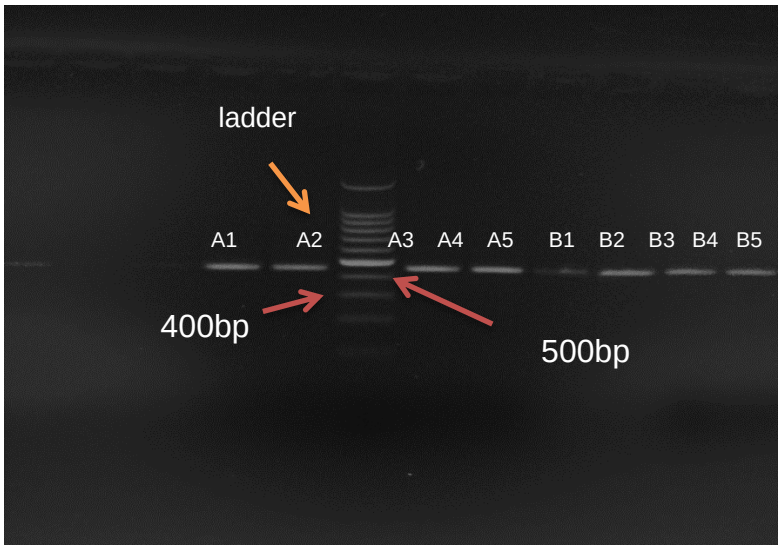


**Fig. 1:** Agarose gel electrophoresis of PCR on extracted DNA from 11 isolates germinal layer (A) & protoscoles (B): with DNA size marker (100-bp DNA ladder) (Promega), in the center image. A1, A2 show no band. Visible bands appear in the other samples.

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