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# Molecular Genotyping of Cystic Echinococcosis in Different Intermediate Hosts using 12 S Mitochondrial DNA Gene

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**Abstract**
In any domestic area, it is important to establish control and prevention strategy for the characterisation of parasite genotype. The purpose of this research is the characterisation of various *Echinococcus granulosus* isolates found in Iraq, by means of the DNA sequence of the mitochondrial 12S rRNA. To conduct the study, naturally infected *Echinococcus granulosus* from thirty lungs and livers of goats, cattle, camel and sheep were gathered from the slaughter- house in Kirkuk city , during the period of February and December 2013. For the genetic work, almost eighteen fertile cysts were used. The technique that is premeditated and tested, required two novel primer sets that precisely amplified the portion of the mitochondrial 12S rRNA gene of 2 *Echinococcus granulosus* genotypes named G6 and G1. One of the two sets of primer augmented a portion of 674 base sets from the G6 genotype. Whereas the second pair amplified a portion of 253 base sets from the genotype G1.
**Keywords**: Echinococcosis, Molecular genotyping, DNA gene

## Introduction

Echinococcosis is a silent zoonotic disease of man and domestic animals, caused by the larval stage of the *E.granulosus* (Satoskar,et al.,2009; Jawetz & delberg's,2007; Parija,2004). Economically and medically, *Echinococcus granulosus* is the prominent zoonoses. Cystic hydatid disease abbreviated as CHD is a long-lasting zoonotic disease. The cause of this disease is the infection that occurs at the larval phase of the tapeworm, found in dogs (Rostami Nejad et al., 2007a; Schantz, 1997; Thompson, 2001). This is a domestic disease in the whole world and it is common in the regions of Mediterranean countries and seas (Euzeby, 1991*).*

The commonest sites of infection are the liver and lungs (Markell et al.,1999) . *Echinococcus granulosus* are categorised into eight core genotypes ranging from G1 to G8. This classification is based on molecular-biological, biological biochemical, and morphological characteristics. Characterisation is made in a way that G1 is the sheep genotype, G3, and G5 are the bovid genotypes, G4 is a horse genotype, G6 represents camelid genotype, G7 represents pig genotype and G8 signifies cervid genotype (Pearson et al., 2002; Bowles et al., 1992). Also, in Poland, a new strain/genotype named G9 is introduced to represent human and pig genotype. The most common and extensive zoonotic genotype is found in *Echinococcus granulosus* (sheep strain, G1) (Craig et al., 1996).

Although the topic seems to be new and emerging still, there are several studies carried on the similar topic. The very first study on this subject was conducted in the year 1998 by Zhang et al. For the analyses of the subject, around sixteen samples of *Echinococcus granulosus* were gathered from domestic animals as well as human cysts. The domestic animals included camels, cattle, goats, and sheep, that were collected from the slaughterhouse in Kirkuk city. Examination method that was used for the study required analyses of sequence variation between nad1 and cox1 genes. The variation was based on the *"polymerase chain reaction-restriction fragment length polymorphism"* (PHR-RFLP) technique. The study concluded that G6 (camel or dog genotype) rarely occurs in Iraq ,while G1 (sheep genotype) are the most common genotypes. It also concluded that in the four examined human isolates, the most common genotype present was G1 (sheep). Also, in domestic animals, G1 (sheep) was more widespread as compared to G6 (camel-restricted) (Zhang et al., 1998).

Characterisation was made on two criteria namely morphological criteria and DNA (*PCR-RFLP of the ITS1 region in the nuclear ribosomal gene cluster*). On the basis of the above-mentioned surveys, new explicit primer sets were designed. For the characterisation of different *Echinococcus granulosus*, these primer sets aim at the mitochondrial 12S rRNA gene. Therefore, a reason to choose this approach is that it is comparatively rapid and straightforward than the PCR-RFLP process utilised before.

Many epidemiological studies were carried out in north of Iraq on the hydatid disease (Amin.,2007; Magid.,2008; Al-Nakeeb.,2004; Abdullah.,2010; Almufty.,2012; Rahimi,etal.,2007). This tudy will open a new clue in strain identification and determination of strains which are infective to human .

## Material and Methods

### 1.1 Sample Processing

A total of 30 liver and lung samples were collected from different domestic animals that included camel, sheep, and cattle. All the specimens were gathered from the Slaughterhouse of Kirkuk city(Animals raised in the city and surrounding areas as well as animals smuggled from neighboring countries).To find the possibility of protoscoleces, an analysis was performed in two ways. First, was the microscopic analysis of flame cell

movement of pure material. The second method was the usage of a vital dye having 0.1% of eosin.

Also, cyst collected was tested separately. It should be noted that in this research protoscoleces are termed as isolates, collected from individual hydatid cyst. However, 3 of the 10, ovine cysts and 9 of the 10, bovine cysts were unfertile, so they were not used in this study. In order to analyse protoscoleces were first aspirated from cysts, Then they were rinsed in saline having fixed ethanol level of about 70%. It should also be noted that volume-by-volume proportion was used for ethanol solution. Also, -20 degrees Celsius temperature was used for the removal and storage of individual protoscoleces from the cyst. Along with DNA extraction, each protoscoleces was cleaned with PBS (phosphate buffered saline) to remove ethanol, used in the previous step. This step was performed numerous times till the ethanol was completely removed.

### 1.2 DNA Extraction

Evaluation of DNA extraction technique for *Echinococcus granulosus* was performed using four distinct approaches that include, a *modified Cinnagen extraction kit* (Cinnagen, Iran), "*modified phenol-chloroform method", "Tissue Extraction Kit"* (Qiagen, Germany), and "*phenol-chloroform*" (Sambrook et al., 1989). The effectiveness of DNA extraction technique was estimated by the extent of achievement in Polymerase Chain Reaction.

#### 2.2.1 Modified phenol–chloroform

Protoscoleses were rinsed with a solution of "*phosphate buffered saline*" (PBS), thrice. Then in 300 ml of protoscoleces, 500 millilitres of lysis buffer having one percent of "*Sodium Dodecyl Sulphate*" (SDS) was added. Afterwards, proteinase K of about 2 millilitres was added and vortexed. Then the mixture was gestated at 55 degrees Celsius for the duration of 2 hours and again at 55 degrees Celsius for approximately 15 minutes. Afterwards, phenol–chloroform–isoamyl alcohol ratio 25:24:1 having a quantity equal to 650 ml was added and gyrated for the duration of 2 mins at 12,000 revolutions per minute. The resulting supernatant obtained was separated and then again chloroform having the same ratio was added, mixed, and spun for the duration of 2 mins at 12,000 revolutions per minute. In the resulting mixture, $1/10^{th}$ the volume of sodium acetate and ethanol having quantity around 500 millilitres were mixed and the tube was kept for thirty minutes at -20 degrees Celsius. Subsequently, it was again gyrated for 10 mins at 12,000 revolutions per minute and the resulting mixture was rinsed with 70 percent ethanol. Afterward, the pellet (sediment particles) was thawed in disinfected super double-purified water for 15 mins at 37 degrees Celsius and then stored at -20 degrees Celsius, until the required PCR is obtained.

#### 2.2.2 Tissue Extraction Kit

Steps for Tissue Extraction kit (Qiagen, Germany) for DNA extraction were followed under the guidelines provided by the manufacturer.

#### 2.2.3 Phenol–chloroform

DNA extraction using phenol-chloroform, for this research followed the guidelines given by Sambrook et al. (1989).

#### 2.2.4 Modified Cinnagen Extraction Kit

For the DNA extraction, first, the DNG™ -Plus mixture (Cinnagen, Iran) was slightly heated with gentle shaking for about 20 mins at the temperature of 37 degrees Celsius. Then 600ml of DNG™ -Plus was combined with 300ml of the sample. The resulting solution is then vortexed for about 20-25 seconds. It should be noted that the sample solution should be wholly homogenous at the end of this step. Afterwards, with the help of vortexing method 450 millilitres (ml) of isopropanol was mixed with the sample obtained from the previous step. In order to keep the concentration of DNA as low as possible, samples of DNA were kept for around 20 mins at the temperature of -20 degrees Celsius. In order to get the supernatant from the sample solution, it was centrifuged for around 10 mins at 12,000 revolutions per minute.

Afterwards, 2 ml of 75% (v/v) of ethanol was further added to the sample, which was then mixed for 3 to 5 seconds by means of vortexing. However, the mixture was again centrifuged for about 5 mins at 12000 revolutions per minute. Likewise, the supernatant came out of the mixture and the pellet (sediment particles) dried out at the temperature of 65 degrees Celsius. Subsequently, the pellet (sediment particles) having DNA was thawed in disinfected purified water having quantity around 50ml.To get the purest form of DNA, the DNA pellet was centrifuged for the last time at 12,000 revolutions per minute again but this time just for 30 seconds. The final step will leave the pure DNA material supernatant as pelting the undissolved material.

### 2. Sequencing

For the sequence of *Echinococcus granulosus* in the GenBank, certain specifications were followed. To specify G1 and G6 genotype accession number AF397617 and AB308063 were specified. Also, for the two genotypes found in Iraq, specific and novel primer sets were premeditated. For the arrangement of G6 and G1 12S rRNA gene categorisations, MegAligne software made by DNASTAR Inc. situated in Madison, USA was used. Likewise, strain-specific primers were considered. With the help of ABI 3130X Genetic Analyser, appropriate

percentages of the 12S rRNA for G6 (674 base pairs) and G1(253 base pairs) were sequenced and amplified.

Figure 1: Represents the arrangement of G6 and G1 12S rRNA sequence.

## 3. Amplification of Genotypes G6 and G1

### 3.1 G1 Genotype

For the G1 genotype, "*Polymerase Chain Reaction*" (PCR) was executed. PCR was executed in a mixture having an overall volume of 25 microliters (µl). The mixture had a 1XPCR buffer, 0.2 millimetres of dNTP (deoxynucleotide triphosphate), 1.5-millimetre $MgCl_2$ (Magnesium Chloride), 1.6 X $10^{-7}$ micrometre of each primer and Ampli-Taq Polymerase having a quantity equal to 0.04 U $\mu l^{-1}$ was used. The primer sets values for G1 reverse are 50-TCA AAC CAG ACATAC ACC AA-30 and G1 forward are 50-GCT TTTGTGTGGATTATGCG-30. To get the PCR product samples amplification was performed for around 35 cycles. First, for 30 seconds denaturation was carried out at 94 degrees Celsius. Then at 57 degrees Celsius annealing was carried out for the 30 seconds. Then the last step required to elongate the mixture at 72 degrees Celsius for around 40 seconds. After amplification, electrophoresis was carried out on 2 percent agarose and 10 microliters of the amplified solution. The resulting solution was later stained with the help of ethidium bromide. In order to confirm the identity, 10 samples of PCR products were sequenced.

### 3.2 G6 Genotype

For the amplification of G6 genotype, primer set G6 reverse (50-TCTAAC TAG CAC AACCTA AT-30) and G6 forward (50-ATT TGT TTATTTATATT AG-30) were used. PCR was executed in a mixture having an overall volume of 25 microliters (µl). The mixture had a 1XPCR buffer, 0.2 millimetres of dNTP (deoxynucleotide triphosphate), 1.5-millimetre $MgCl_2$ (Magnesium Chloride), 1.6 X $10^{-7}$ micrometre of each primer and Ampli-Taq Polymerase having a quantity equal to 0.04 U $\mu l^{-1}$ was used.

Also, here amplification was performed for 35 cycles. First, for 30 seconds denaturation was carried out at 94 degrees Celsius. Then at 59 degrees Celsius annealing was carried out for the 30 seconds. Then the last step required to elongate the mixture at 72 degrees Celsius for around 40 seconds. After amplification, electrophoresis was carried out on 2 percent agarose and 10 microliters of the amplified solution. The resulting solution was later stained with the help of ethidium bromide. In order to confirm the identity, 10 samples of PCR products were sequenced.

```
G1   GTTTGTTTAATTGTGGCAATAGGAAGTTTTGTTTCATTGACTGCATGAAGTGTGTTTAT-  59
     |||||||||| |||||||||| |||||||||||||||| |||||||| | |||||||
G6   GTTTGTTTAACTGTGGCAATAGAAAGTTTTGTTTCATTGGCTGCATGAAATTTGTTTATT  61

G1   ---GATTTGTGGGTTATGTTTTCTGGTTATTTTATCAGCAGTTGGTTTTTTGTTAAGATG  116
        |||| ||  |||||||||||||| |||||||||||||||||| |||||||||||||
G6   TATGATTAGTTTGTTATGTTTTCTGATTATTTTATCAGCAGTTGATTTTTTGTTAAGACG  121

G1   AGAATCTTATAAGGGTGATACAATTTGAGGGGATAGGTCACAGTGCCAGCATCTGCGGTT  176
      || |||||||||||||| |||||||||||||||| |||||||||||||||||||||||
G6   TGAGTCTTATAAGGGTGATGCAATTTGAGGGGATAGGACACAGTGCCAGCATCTGCGGTT  181

G1   AATCTGTTTTCTTTGCTTTTGTGTGGATTATGCGTATTTATTTATGTGCAATGTTAGGTT  236
     | |||||||||||||||||| ||||||| ||||| ||||||||| | ||||||||||||
G6   AGTCTGTTTTCTTTGCTTTTATGTGGATAGTGCGTGTTTATTTATATTCAATGTTAGGTT  241

G1   TTACTCTTTTTATGTTGGTGTATGTCTGGTTTGATATTATTGTTGAATAATTTAAGTTTG  475
     | |||||| ||||||||||| |||||||||||||||| ||| |||  ||| ||||||
G6   TCACTCTTGTTATGTTGGTGTATATCTGGTTTGATATTATCGTTTAATGGTTTGAGTTTG  481

G1   AGGTAAGTCGTAACAAGGTAACTTTAAATGAATTTGAAGTTAATTATTAGGTTGTCCTAG  714
     |||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||
G6   AGGTAAGTCGTAACAAGGTAACTTTAAATGAATTTGAAGTTAATTATTAGGTTGTGCTAG  721

G1   TTGTA  719
     ||  |
G6   TTAGA  726
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**Figure 1:** Arrangement of 12S rRNA nucleotide orders belonging to G6 (AB308063) and G1 (AF397617) strains. Underlined regions represent the primer set design. To amplify the outer part of G1 genotype and G6 genotype, outer sets of primers were designed. Numbering for 12S gene is done on the basis of observations in the GenBank. In G1 genotype, 12S gene ends at 700 as per the annotation in AF297617.

## 4. Results

This study concludes that as compared to other methods, phenol-chloroform and Cinnagen extraction kit techniques, both give similar results and produces high-quality DNA. With specific bands of 674 base pairs (figure.3) and 253 base pairs (figure.2), G6 and G1 PCR primer set carefully amplified the genotypes G6 and G1 of Echinococcus granulosus. The G6 primer set did not yield any sort of specific results in PCR utilising G1 genotype of DNA and same goes for the G1 primer set.

The eighteen fertile cysts taken from 10 goats, 7 sheep and 1 cattle yielded comparable bands with the novel G1 strain. Efforts were also made to produce similar results using G6 genotype but no products were detected. While performing public databases searches using NCBI-BLAST, it was revealed that all the 10 sequences obtained from the fertile cyst, belonged to G1 genotype.

It has been found that G6 primer sets only amplified selective G6 referral specimen of Echinococcus granulosus, producing a band having base pairs equal to 676 bp (figure.3). Also, Sequences retrieved from the specimen named "EU541210" were found to be identical to the ones in G6 genotypes present in GenBank.

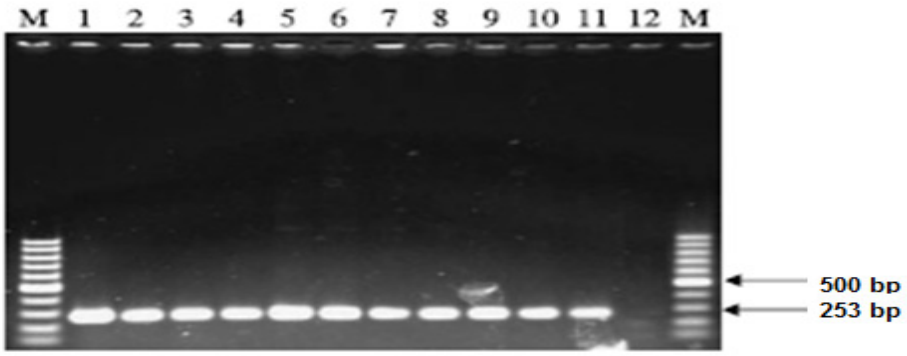


**Figure 2:** G1 genotype of Echinococcus granulosus generating gel electrophoresis of the PCR products (lane 1 to 11) using 2 percent agarose gel. However, Lane 12 represents negative control value; M lanes and hundred base pair indicators are used.

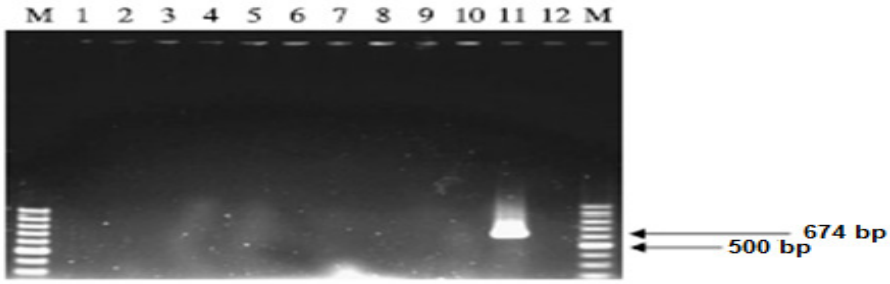


**Figure 3:** Generation of Echinococcus granulosus isolates from PCR sample's gel electrophoresis, amplified with the help of G1 primers, utilized along with G6 primer sets for G6 strains (lane 1 to 10) using 2% agarose gel. When amplified with G6 primers, samples produced a band of 674 base pairs. Lane 12 represents negative control while lanes are represented via M. Also, 100 base pair markers are used.

## 5. Discussion

For classification at the level of species, genes, and sub-species, DNA-based techniques are found to be useful. However, the technique requires a lot of attention for designing primers and preparing purest DNA in suitable quantities (Rahimi et al., 2007; McManus & Thompson, 2003). In Iraq, there are previous successful researches on molecular studies on Echinococcus granulosus using mitochondrial and nuclear DNA analyses. The research conducted by Abdullah et al(2012) used DNA sequence variation technique that accessed PCR-RFLP. However, the study was carried in the areas of cox1 genes of *Echinococcus granulosus*. The study showed the emission of two types genotypes G6 and G1. Also, the research conducted by Fasihi Harandi et al in the year (2002), yielded similar results, although, the technique used was molecular characterisation and morphological features of the ITS1 region of rDNA. Another study conducted by Ahmadi and Dalimi (2006), found 2 common genotypes amongst camel, human and sheep isolates. They also used the technique of focusing ITS1 region of the rDNA.

In the current study, procedures for extraction of DNA were improved. Also, based on the sequences of *Echinococcus granulosus* specific genotype primer sets in the 12S rRNA gene were designed. The results

obtained were specific and gene sequences confirmed our study when compared to the samples in the GenBank. The impact on public well-being can be rapidly and easily notable from these specific primer sets by utilising target sequence as a portion of the mitochondrial 12S rRNA gene. Genotypes G6 and G1 can easily be identified with the help of specific PCR as discussed in the research.

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