Molecular and phylogenetic studies of *Taenia hydatigena* cysts in native sheep in Sulaimani Province, Iraq

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**ABSTRACT**

This study aimed to determine the strain(s) of *Taenia hydatigena* parasite in native breed of sheep in Sulaimani province of Iraq by using polymerase chain reaction, DNA sequencing and phylogenetic analysis. Sheep isolates (20) of *T. hydatigena* cysts were collected from native breed of sheep at Modern Sulaimani Slaughterhouse. Following DNA extraction, fragment of cytochrome c oxidase 1 (cox1) gene was amplified by the polymerase chain reaction using specific primer and 4 highly concentrated amplicons were subjected to partial sequencing. All isolates were 100% identical, indicating no genetic variation in isolates. Phylogenetic analysis computed by neighbor joining (NJ) from the partial cox1 gene nucleotide sequences revealed that the Iraqi *T. hydatigena* isolates identified in this study were clustered in one clade, along with isolates from Mongolia and China. It can be concluded that the identified strain from sheep isolates was similar to the Mongolian and Chinese strains, and closely related to strains of neighboring countries especially Iran and Turkey.

**Key words**: PCR amplification, Phylogenetic analysis, *Taenia hydatigena* cyst

Ovine cysticercosis caused by the larval stage of *T. hydatigena* is a universal and widespread disease, which has economic and animal health significance (Kassai 1999). The mature tapeworm of *T. hydatigena* belongs to the family Taeniidae which inhabited in the small intestine of carnivorous, while the metacestode (Cysticercus tenuicollis) of this cestode is harbored as a fluid-filled cyst in the abdominal, pleural and pelvic cavities of domestic and wild ruminants (Kassai 1999, Murrell et al. 2005). The massive number of migrating living cysticerci in the liver parenchyma of intermediate hosts, create “hepatitis cysticercosa”, which is frequently leads to death of young animals (Kara and Doganay 2005, Taylor et al. 2016).

Generally, the recognizable proof of taenids has been found on morphological criteria, typically considering likewise natural and organic perspective like host specificity (Abuladze 1964). The accurate diagnosis and identification of taeniosis and cysticercosis are based on the molecular analysis of the parasite (Kassai 1999, Gonzalez et al. 2006, McManus 2006). Different molecular approaches to differentiate *Taenia* to their species have been developed, including restriction fragment length polymorphism (RFLP) analysis, PCR-linked RFLP analysis (PCR-RFLP), and direct comparison of PCR-amplified DNA sequences (Gasser et al. 1999, Murrell et al. 2005, Gonzalez et al. 2006, McManus 2006).

Mitochondrial DNA sequence data have been widely used as genetic markers to examine the population genetic structures of animals, including taeniid cestodes, as it experiences low recombination rates. These sequences have proven useful for not only studying evolutionary relationships among distantly related taxa, but also for species differentiation of parasitic flatworms (Lavikainen et al. 2008, Liu et al. 2012, Li et al. 2013). The cox1 gene has been found to be useful population genetic marker for *Taenia* and many cox1 gene sequence data is available on GenBank (Utukand and Piskin 2012). Specific identification and characterization of the members of family Taeniidae are fundamental for vaccine development, precise diagnosis, treatment, and effective control programs of these cestodes (McManus 2002).

The purpose of this study was to determine the strain(s) of sheep isolate of *T. hydatigena* by PCR amplification of cox1 gene and partial sequencing of mt-cox1 gene in Sulaimani province of Iraq.

**MATERIALS AND METHODS**

Sample collection and preparation: Twenty specimens of *T. hydatigena* cysts (*C. tenuicollis*) were collected from native breed of sheep during routine meat inspection at Modern Sulaimani Slaughterhouse located in Sulaimani...
proportion of Iraq from March to April 2017. The cysts were identified initially based on their morphological features such as semi-transparent cyst fluid with a long-necked single scolex (Taylor et al. 2016). The host tissue surrounding the collected cysts was removed manually and the cysts disinfected with 70% ethanol, then washed thoroughly in normal saline, transferred into sterile containers, and stored at −20°C, until the preparing of genomic DNA extraction (Braa et al. 2015).

**Genomic DNA analysis:** From each individual cysticercci collected, total genomic DNA was isolated from a quarter of scolex and a piece of the larval stage membrane using PrimePrep Genomic DNA Extraction kit (from tissue). Briefly, the tissues were mixed with 200 µl tissue lysis buffer (TL buffer) and 20 µl proteinase K and incubated at 56°C for approximately an hour until the specimens were lysed. According to the manufacturer’s instructions, DNA was extracted using ethanol and buffers then eluted with 200 µl elution buffer (TE) provided by the protocol of the company (GeNet Bio Co., Daejeon, Korea). DNA concentration was evaluated using Genova Nano Spectrophotometer (Jenway, UK) and the purity of the samples ranged between 21-51 ng/µl.

**PCR assay and sequencing of amplicons:** To amplify the fragment of the cox1 gene from *T. hydatigena* cysts, a pair of primers including A1 (forward): 5′-TGGGTAGAGTGTTGGGGT-3′, A2 (reverse): 5′-TTCCATACACACAACGC-3′ were designed from GenBank accession number (NC_012896), using Primer 3 (http://primer3.wi.mit.edu/). On the other hand, A2 (reverse) and JB4.5 (reverse) primers were used for sequencing the amplicons. The JB4.5 primer (5′-TTAAGAAAGACATATGAAAATG-3′) has been previously established by Bowles et al. (1992).

Conventional PCR was performed independently for each specimen in 20 µl reaction containing 1× Prime Taq Premix (2 µl) which contains Prime Taq DNA polymerase 1 unit, 2 µl reaction buffer, 4 mM MgCl2, enzyme stabilizer, sediment, loading dye, pH 9.0 and 0.5 mM each of dATP, dCTP, dGTP, dTTP, 5 µl of DNA template and 1 µl final concentration of scolex and a piece of the larval stage membrane using PCR cycler (Techne, UK). The PCR products were denatured for 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C (denaturation), 30 sec at 55°C (annealing), and 30 sec at 72°C (extension). Finally, the reactions were incubated for 5 min at 72°C (final extension) using a thermal cycler (Techne, UK).

About 5 µl of the amplification products were run at 110 V for 70 min on a 1% agarose gel in 1× TBE (87.5 mM Trisbase, 89 mM boric acid, 3 mM EDTA) and stained with ethidium bromide (Bio Basic, Canada).

Among the PCR products, four highly concentrated DNA were chosen for partial sequencing. Purification and sequencing techniques were carried out by Macrogen Co., Seoul, Korea. A total of 5 µl of amplicons from each samples and 5 µl (10 pmol/µl) of reverse primer (A2) or reverse JB4.5 for reverse sequencing, respectively were sequenced using the Sanger method by capillary DNA analyzer (ABI 3730XL; Applied Biosystems, Japan) and edited by CodonCode Aligner (CodonCode Corporation).

**Phylogenetic construction:** PCR products of cox1 gene (four isolates) were sequenced partially using Sanger sequencing. All gene sequences have been deposited in NCBI GenBank using Bankit (Benson et al. 2015) and their accession numbers are MH113919-MH113922.

For the phylogenetic analysis, all sequences were analysed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the obtained sequences with sequences from GenBank. The cox1 gene sequences together with those from representative strains were aligned with CLUSTAL W software and examined using the program MEGA (Molecular Evolutionary Genetics Analysis) version 7. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with the distance algorithms available in MEGA version 7. The distances were calculated using the Kimura 2-parameter method. The robustness of the tree topology was examined using bootstrap analysis. Bootstrap values were determined with 1000 replicates of the data sets (Kumar et al. 2016). For comparison, sequences of *T. hydatigena, T. asiatica, T. krabbei, T. multiceps, T. musteale, T. ovis, T. pisiformis, T. saginata, T. serialis, T. solium, T. taeniaeformis* and *E. granulosus* from GenBank were included in the analysis with accession numbers, host and country origins (Table 1).

### Table 1. Sequences from GenBank used for genetic comparison

| Species            | Country | Host | GenBank accession no. |
|--------------------|---------|------|-----------------------|
| *T. hydatigena*     | Mongolia | Dog  | AB792724              |
| *T. hydatigena*     | China   | Goat | JN831298              |
| *T. hydatigena*     | Iran    | Goat | KR337823              |
| *T. hydatigena*     | Mongolia | Dog  | AB792721              |
| *T. hydatigena*     | China   | Goat | JN831300              |
| *T. hydatigena*     | China   | Dog  | HX204205              |
| *T. hydatigena*     | India   | Sheep| DQ999566              |
| *T. hydatigena*     | Peru    | Camel| KP878693              |
| *T. hydatigena*     | Turkey  | Dog  | KP268023              |
| *T. hydatigena*     | Turkey  | Goat | JN827307              |
| *T. asiatica*       | Thailand| Human| JQ517298              |
| *T. krabbei*        | Canada  | Wolf | KX508184              |
| *T. multiceps*      | China   | Goat | KX547633              |
| *T. musteale*       | Japan   | –    | AB732960              |
| *T. ovis*           | Denmark | Sheep| MG594802              |
| *T. pisiformis*     | China   | –    | JN870104              |
| *T. saginata*       | Laos    | Rabbit| KX290373              |
| *T. serialis*       | China   | Rabbit| KY007158              |
| *T. solium*         | China   | Human| AB984356              |
| *T. taeniaeformis*  | Japan   | –    | AB745097              |
| *E. granulosus*     | Portugal| Cattle| HF947565              |

### RESULTS AND DISCUSSION

The cox1 gene has been reported as useful targets for molecular characterization of *C. tenuicollis* species. Molecular characterization of a specific gene is important
to indicate a gene variation according to different geographical areas which may affect the pathogenesis of the infectious agent. In *C. tenuicollis*, cox1 gene is an important tool to detect the existence of any mutation and gene variation and it is globally confirmed because this region is highly conservative region among taeniid. Several studies were published in different countries and they characterized the gene sequence and variation, but the case in Sulaimani province of Iraq is still unknown. The recent research was carried out in Turkey and gene variation was compared to the study finding of China and India with the existence of some new mutations and 99% of gene sequence identity.

In this study, the cox1 gene was amplified by PCR using
specific primers to find the gene diversity. For the purpose of gene amplification, DNA extraction was carried out for 20 cysts of *T. hydatigena*. PCR amplification was successfully obtained (488 bp) on all of the isolates.

Among the PCR products, four highly concentrated DNA were chosen for partial sequencing and we obtained 4 identical gene sequences that could be available in the NCBI under accession numbers MH113919-MH113922. The nucleotide sequence of this study was aligned and compared with published sequence results of three countries, viz. India (DQ995656), China (HQ204206), and Turkey (JN827307) for *T. hydatigena* (Fig. 1). According to the alignment results, there was 99% identity in nucleotide sequences for *T. hydatigena*.

Consequently, a phylogenetic relationship was carried on cox1 sequence data to illustrate the likenesses and contrasts of the *T. hydatigena* genotypes. Phylogenetic tree compare the *T. hydatigena* cysts obtained from the current study with other GenBank accession numbers of *T. hydatigena* and other taeniid (Fig. 2). A similar topology of cox1 phylogenetic tree of our isolates and Mongolian *T. hydatigena*, along with isolates from China was observed, however, the general topology of the cox1 tree was different from other taeniid isolates. The low bootstrap values indicated that the topology within the *T. hydatigena* clade was not robust, reflecting low genetic variability among *T. hydatigena* strains.

Mt-DNA is generally utilized in molecular and phylogenetic investigations of eukaryotes because of its low or lack of recombination, maternal inheritance, absence of introns, highly conserved structure, higher rate of mutation and a moderately rapid evolutionary rate (Moritz *et al.* 1987, Avise 2000).

By amplification of PCR product (488 bp) and sequence analysis of the cox1 gene obtained in this study, all analysed cysts were found to be *T. hydatigena*, confirming the gross Figure 2. Phylogenetic analysis of cox1 gene sequences among *Taenia* species. The scale bar represents 0.02% divergence. Bootstrap values are shown above or below branches. MH113919-MH113922 GenBank accession numbers represent *T. hydatigena* sequences identified in this study.
inspection. The cluster analysis of *T. hydatigena* and other species of taeniids suggested that cox1 is a good target gene for identifying *T. hydatigena*, *T. ovis*, *T. taeniaeformis*, *T. pisiformis*, *T. multiceps*, *T. serialis*, *T. solium*, and *T. saginata* and possibly other taeniids as well (Braae et al. 2015).

On gel electrophoresis, out of 20 positive samples, sequences of 4 samples showing strong signals were determined, and all the *Taenia* parasiates were identified as *T. hydatigena* with 100% similarity with the *T. hydatigena* strain Th04 in Mongolia (Accession no. AB792724) and *T. hydatigena* strain Panzhihua8 in China (Accession no. JN831298). The results of sequence analysis of our study showed that all 4 isolates were 100% identical, suggesting that there was no genetic variation of our isolates. This might be due to smaller geographical area and less dispersed population of livestock in study region. This is an agreement with the results of study conducted in Argentina (Kamenetzky et al. 2000), who reported that 7 isolates of *E. granulosus* were completely identical. While, disagreement with that reported in Mongolia, China, and Iran (Rostami et al. 2015). In addition, slight mutation and micro variation had been found when comparing sequence of our study with published sequences of India (DQ995656), China (HQ204206), and Turkey (JN827307). This may be due to geographical factor.

To the best of our knowledge, this is the first report on phylogenetic construction regarding to *T. hydatigena* cysts in Iraq. The phylogenetic data revealed that *T. hydatigena* strain in Sulaimani province of Iraq was closely related to the Mongolian Th04 strain, Chinese strain Panzhihua 8, and *T. hydatigena* strains of other countries especially Iran and Turkey. This outcome is not astonishment as the region shares its border with Iran in which the genetic diversity is very low compared to Iran. Because of commercial relationship between Iran and China, as well as with Iraq it might be possible that the origin of *T. hydatigena* had been found in China. The findings of the current study are essential to understand the epidemiology and to perform future planning control program against the identified parasite in Sulaimani province of Iraq.

In this study, we recorded the *T. hydatigena* strain in sheep in Iraq for the first time using PCR technique and partial sequencing of mt-cox1 gene. Furthermore, phylogenetic analysis revealed that all the examined isolates of *T. hydatigena* cyst were 100% identical and the recognized parasite was similar to the Mongolian and Chinese strains.

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