



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

ARAM AHMAD MOHAMMED¹ and MOHAMMED ABDUL-AZIZ KADIR²

Sulaimani University, Kurdistan, 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 taeniids 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

Present address: ¹Lecturer (aram.mohammed@univsul.edu.iq), Department of Microbiology, College of Veterinary Medicine. ²Professor (mohammdsalam@yahoo.com), Department of Microbiology, College of Medicine, Kirkuk University, Iraq.

province 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 (Braae *et al.* 2015).

Genomic DNA analysis: From each individual cysticerci 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' TGGGTAGAAGTGTGTGGGGT-3', A2 (reverse): 5'TTCATACACAAACACGCCG-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'-TAAAGAAAGAACATAATGAAAATG-3') has been previously established by Bowles *et al.* (1992).

Conventional PCR was performed independently for each specimen in 20 μl reaction containing 1 $\times$ Prime TaqPremix (2 μl) which contains Prime Taq DNA polymerase 1 unit, 2 μl reaction buffer, 4 mM MgCl_2 , 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 from each of the A1 and A2 primers. The DNA were initially 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 $\times$ 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.
<i>T. hydatigena</i>	Mongolia	Dog	AB792724
<i>T. hydatigena</i>	China	Goat	JN831298
<i>T. hydatigena</i>	Iran	Goat	KR337823
<i>T. hydatigena</i>	Mongolia	Dog	AB792721
<i>T. hydatigena</i>	China	Goat	JN831300
<i>T. hydatigena</i>	China	Dog	HQ204205
<i>T. hydatigena</i>	India	Sheep	DQ995656
<i>T. hydatigena</i>	Peru	Camel	KP878693
<i>T. hydatigena</i>	Turkey	Dog	KF268023
<i>T. hydatigena</i>	Turkey	Goat	JN827307
<i>T. asiatica</i>	Thailand	Human	JQ517298
<i>T. krabbei</i>	Canada	Wolf	KX058184
<i>T. multiceps</i>	China	Goat	KX547633
<i>T. musteale</i>	Japan	—	AB732960
<i>T. ovis</i>	Denmark	Sheep	MG594802
<i>T. pisiformis</i>	China	—	JN870104
<i>T. saginata</i>	Laos	Human	KY290373
<i>T. serialis</i>	China	Rabbit	KY007158
<i>T. solium</i>	China	Human	AB984356
<i>T. taeniaeformis</i>	Japan	—	AB745097
<i>E. granulosus</i>	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

		20		40		60	
DQ995656	GGATTTGGAA	TTATTAGTCA	TATATGTTTG	AGAATAAGCA	TGAGTCCTGA	TGCTTTTGGGA	60
HQ204206	GGATTTGGAA	TTATTAGTCA	TATATGTTTG	AGAATAAGTA	TGAGTCCTGA	TGCTTTTGGGA	60
JN827307.1	GGATTTGGAA	TTATTAGTCA	TATATGTTTG	AGAATAAGCA	TGAGTCCTGA	TGCTTTTGGGA	60
MH113919	GGATTTGGAA	TTATTAGTCA	TATATGTTTG	AGAATAAGCA	TGAGTCCTGA	TGCTTTTGGGA	60
	*****	*****	*****	*****	*****	*****	
		80		100		120	
DQ995656	TTCTATGGAT	TATTATTTGC	TATGTTTTC	ATAGTCTGTT	TGGGTAGAAG	TGTGTGGGGT	120
HQ204206	TTCTATGGAT	TATTATTTGC	TATGTTTTC	ATAGTCTGTT	TGGGTAGAAG	TGTGTGGGGT	120
JN827307.1	TTCTATGGAT	TATTATTTGC	TATGTTTTC	ATAGTCTGTT	TGGGTAGAAG	TGTGTGGGGT	120
MH113919	TTCTATGGAT	TATTATTTGC	TATGTTTTC	ATAGTCTGTT	TGGGTAGAAG	TGTGTGGGGT	120
	*****	*****	*****	*****	*****	*****	
		140		160		180	
DQ995656	CATCATATGT	TTACTGTTGG	ATTAGATGTT	AAGACTGCTG	TTTTTTTTAG	TTCTGTCAC	180
HQ204206	CATCATATGT	TTACTGTTGG	ATTAGATGTT	AAGACTGCTG	TTTTTTTTAG	TTCTGTCAC	180
JN827307.1	CATCATATGT	TTACTGTTGG	ATTAGATGTT	AAGACTGCTG	TTTTTTTTAG	TTCTGTCAC	180
MH113919	CATCATATGT	TTACTGTTGG	ATTAGATGTT	AAGACTGCTG	TTTTTTTTAG	TTCTGTCAC	180
	*****	*****	*****	*****	*****	*****	
		200		220		240	
DQ995656	ATGATTATAG	GTGTGCCTAC	TGGTATAAAG	GTGTTTACTT	GTTTATATAT	GCTTTTAAAC	240
HQ204206	ATGATTATAG	GTGTGCCTAC	TGGTATAAAG	GTGTTTACTT	GTTTATATAT	GCTTTTAAAC	200
JN827307.1	ATGATTATAG	GTGTGCCTAC	TGGTATAAAG	GTGTTTACTT	GTTTATATAT	GCTTTTAAAC	200
MH113919	ATGATTATAG	GTGTGCCTAC	TGGTATAAAG	GTGTTTACTT	GTTTATATAT	GCTTTTAAAC	200
	*****	*****	*****	*****	*****	*****	
		260		280		300	
DQ995656	TCTCATGTGA	ATAAGAGTGA	TCCTGTTGTT	TGATGAATTG	TTTCTTTTAT	AGTTTTGTTT	300
HQ204206	TCTCATGTGA	ATAAGAGTGA	TCCTGTTGTT	TGATGAATTG	TTTCTTTTAT	AGTTTTGTTT	300
JN827307.1	TCTCATGTGA	ATAAGAGTGA	TCCTGTTGTT	TGATGAATTG	TTTCTTTTAT	AGTTTTGTTT	300
MH113919	TCTCATGTGA	ATAAGAGTGA	TCCTGTTGTT	TGATGAATTG	TTTCTTTTAT	AGTTTTGTTT	300
	*****	*****	*****	*****	*****	*****	
		320		340		360	
DQ995656	ACTTTTGGTG	GGGTTACTGG	TATTGTGTTG	TCAGCATGTG	TATTAGATAA	AGTTC TTCAT	360
HQ204206	ACTTTTGGTG	GGGTTACTGG	TATTGTGTTG	TCAGCATGTG	TATTAGATAA	AGTTC TTCAT	360
JN827307.1	ACTTTTGGTG	GGGTTACTGG	TATTGTGTTG	TCAGCATGTG	TATTAGATAA	AGTTC TTCAT	360
MH113919	ACTTTTGGTG	GGGTTACTGG	TATTGTGTTG	TCAGCATGTG	TATTAGATAA	AGTTC TTCAT	360
	*****	*****	*****	*****	*****	*****	
		380					
DQ995656	GATACCTGGT	TTGTAGTTGC	TCATTTTCAT	TATGTT			396
HQ204206	GATACCTGGT	TTGTAGTTGC	TCATTTTCAT	TATGTT			396
JN827307.1	GATACCTGGT	TTGTAGTTGC	TCATTTTCAT	TATGTT			396
MH113919	GATACCTGGT	TTGTAGTTGC	TCATTTTCAT	TATGTT			396
	*****	*****	*****	*****			

Fig. 1. Nucleotide sequence of partial mt-cox1 for *T. hydatigena* cysts in the current study (MH113919) aligned with the published (DQ995656, HQ204206, JN827307) mt-cox1 sequences of the *T. hydatigena* as a reference. An asterisk indicates a nucleotide that is conserved relative to the published sequence.

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

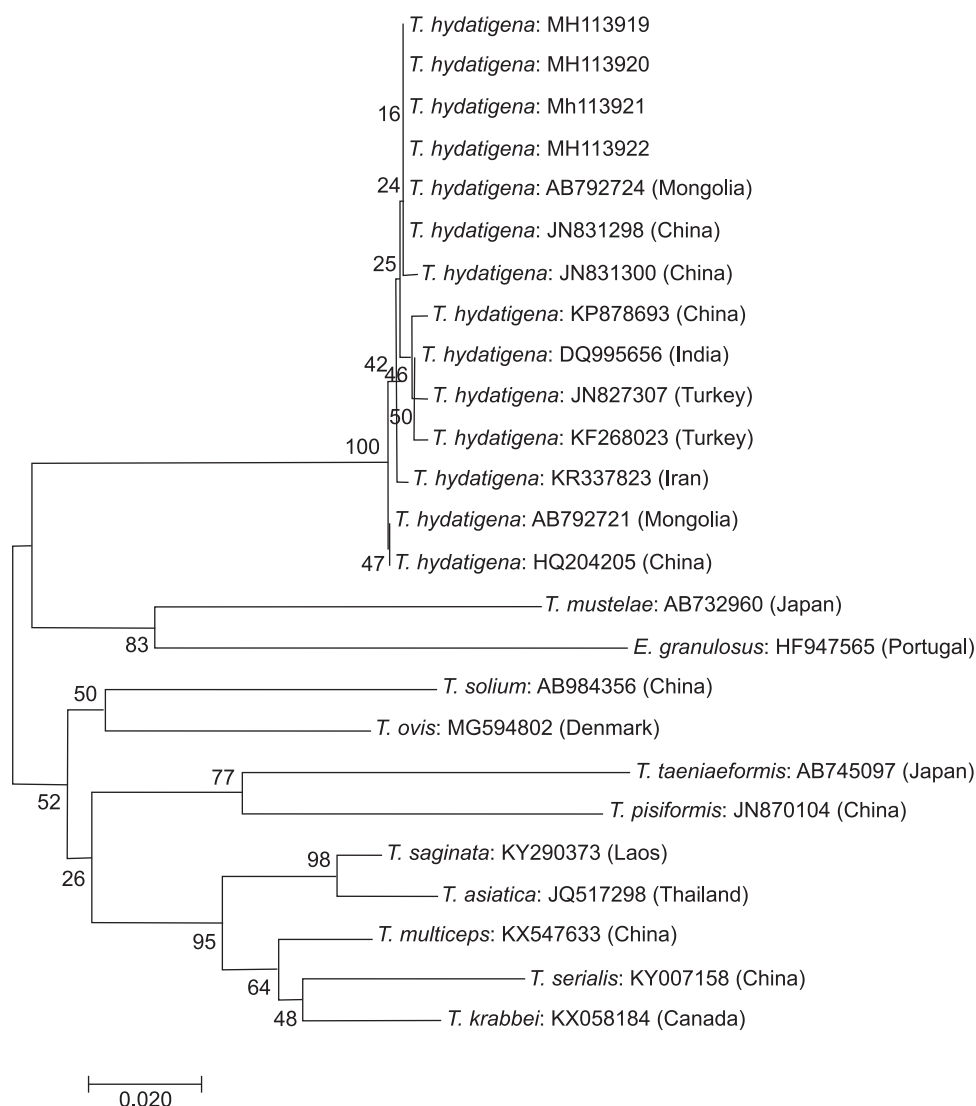


Fig. 2. Phylogenetic analysis of *cox1* gene sequences among *Taenia* species. The scale bar represents 0.02% divergence. Bootstrap values are shown above or below branches. MH113919-MH113922 GenBank accession numbers represent *T. hydatigena* sequences identified in this study.

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

inspection. The cluster analysis of *T. hydatigena* and other species of taeniids suggest 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* parasites 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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