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Molecular Characterization and Phylogeny of *Taenia hydatigena* and *Echinococcus granulosus* from Iranian Sheep and Cattle Based on *COX1* Gene

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Abstract

Hydatid cyst, the larval stage of *Echinococcus granulosus*, and *Cysticercus tenuicollis*, the larval stage of *Taenia hydatigena*, are prevalent in domestic, livestock, and wild ruminants. The main goal of this research was to identify the isolates of *E. granulosus* and *C. tenuicollis* by partial sequencing with PCR amplification of the cytochrome C oxidase 1 (*COX1*) gene. During a routine veterinary inspection at a Chabahar city slaughterhouse, two samples of *hydatid cysts* from sheep's liver and cattle's lung and two samples of *C. tenuicollis* from sheep's liver were collected. After DNA extraction, the fragment of the *COX1* gene was amplified by the PCR method. Sample sequences were modified and synchronized by Chromas and CLC genomic workbench 11 software. Sequence analysis was carried out by BLAST algorithms and GenBank databases. Phylogenetic trees were performed using MEGA 7 software and the neighbor-joining and maximum likelihood method for *T. hydatigena* and *E. granulousus*. The result indicated that the main genotype of parasites and the amplified fragment size were G1 and approximately 455 bp, respectively. The analysis of phylogenetic trees based on nucleic acid for four samples showed that there was a common ancestor. However, the shift in nucleotides in the two isolates in *E. granulosus* and the two isolates of *T. hydatigena* were non-synonymous type and synonymous type, respectively. The present study showed that the dominant genotype in all isolates was G1 and this report was similar to other studies in Iran and the world. Also, the partial *COX1* gene sequence was matched with *T. hydatigena*.

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Introduction

Hydatidosis is a zoonotic parasitic disease caused by *Echinococcus granulosus* tapeworm; the infective stage for this disease is the larval stage. The infection is widely distributed, especially in developing countries [1–3]. The prevalence of that disease varied according to climate and contact with sheep [4]. In Iran, the incidence rates of *Hydatid cyst* have been detected in different livestock such as sheep (5.1–74.4%), camels (25.7–59.3%), cattle (3.5–38.3%), and goats (2–20%) [5–7]. Annually, 1.27 per hundred thousand of the infected human resort to surgery; 2.5% out of them has been died [8]. In Iran, various investigating techniques have been conducted for *E. granulosus* identification and genotyping by using mitochondrial *COX1* and Nad1 genes [9, 10]. The investigations showed 10 genotypes for this parasite which were recognized as G1 to G10 [9]. G1 has been organized as the most prevalent genotype in Iran and globally [11]. *T. hydatigena* is a global zoonotic intestinal parasite, which infects a wide range of carnivores and livestock

including sheep, buffalo, yak, cattle, and goats, the infective stage of this disease called *Cysticercus tenuicollis* [12, 13]. *T. hydatigena* prevalence in Iran ranges from 6 to 80% (in dog), 12.86% (in sheep), and 18.04% (in goat), respectively [14–16]. The infection started by ingestion of the eggs, which hatch in the intestine to release oncospheres. The oncospheres migrate to the abdominal cavity via the bloodstream [17, 18]. Cysticercosis diagnosis is based on microscopic examination to detect the morphological features, such as blade length and hook number, length. Recently, molecular diagnosis is widely used to identify and differentiate between different parasites [13, 19, 20]. Few studies were conducted for studying the genetic variation of *T. hydatigena* by using *COX1 and Nad1* mitochondrial gene sequences among different populations and geographical regions [18, 19, 21–26]. Therefore, the present study aimed to identify the genetic variation of *E. granulosus* and *T. hydatigena* in Chabahar, Sistan, and Baluchestan Province, Iran, based on the *COX1* gene and infer the phylogenetic tree of *E. granulosus* and *T. hydatigena*.

Materials and Methods

Study Area

Chabahar is located on the Makran Coast of Iran's Province of Sistan and Baluchestan and has dry, humid summer weather, and mild winter weather, which gives it a hot desert climate. The summer monsoon winds from the Indian subcontinent make Chabahar the coolest southern port in the summer and the warmest part of Iran in the winter. It has an average maximum and minimum temperature of 34 °C and 21.5 °C, respectively [27].

Samples Collection and Diagnosis

During a routine veterinary inspection at a Chabahar city slaughterhouse, two samples of *hydatid cysts* (larvae of *E. granulosus*) from sheep's liver and cattle's lung and two samples of *C. tenuicollis* (larvae of *T. hydatigena*) from sheep's liver were collected. The process of morphological diagnosis of all samples was performed by specialists in the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences. The endocystic cystic cervical wall of *C. tenuicollis* and the germinal layer of *the hydatid cyst* were examined and then washed several times with a 9% solution of calcium chloride (Fig. S1). The calyceal cyst fluid and the endocysts were evacuated (Fig. S2). All samples were preserved in 70% ethanol at -20 C until DNA extraction.

DNA Extraction

A commercial kit (AccuPrep ® Genomic DNA Extraction Kit, Cat. No. K-3032-bioneer Korea) was used to extract genomic DNA from the germinal layer of the *hydatid cyst* and the endocystic cervical wall of *C. tenuicollis*. All samples extracted were blended with 80 µl of proteinase k, and the samples were placed in an incubator at 56 °C for 24 h on the Kit's Protocol concept. After this step, DNA samples were stored at – 20 °C until the PCR stage.

PCR

To estimate transcription levels, PCR was carried out using a transcript Fermentas PCR Kit employing the following cycling protocol: 95 °C/300 s and 94 °C/30 s followed by 35 cycles of 50 °C/45 s, 72° C /35 s, and 72 °C /600 s. JB3-F (5-TTTTTTGGGCATCCTGAGGTTTAT-3) and JB4.5-R (5-TAAAGAAAGAACATAATGAAAATG-3) sequences were used as *COX1* gene forward and reverse primers. The amplified products were eventually stained by simple safe (EURx, Poland) and separated in the TAE buffer by electrophoresis on %1 agarose gel and visualized under a transilluminator. Positive control used (Accession Number: scox1-2 (KT033487) [28].

Sequencing and Phylogenetic Analysis

Positive amplicons were purified and sequenced by the Iranian Takapozist Company and South Korea (Bioneer), respectively. The nucleotide sequences were revised and edited using Chromas version 2.4, and CLC genomic workbench 11 software and compared to sequences from genomic databases with BLAST. The obtained data were aligned by the reference genotypes of *E. granulosus* and *T. hydatigena* in Gene bank to determine the genotypes using CLC genomic workbench 11 software, and the drawing of the tree was performed using MEGA7 software. Trees plotted with two methods of neighbor-joining and maximum likelihood based on nucleic acid and amino acid for *E. granulosus* and *T. hydatigena*.

Results

Genotyping of *E. granulosus* and *T. hydatigena* in Iran

The PCR amplification products showed a 45-5 bp fragment of the *COX1* gene and the gene sequence compared to the sequences in the Gene bank (Fig. S3). The result indicated

that the main genotype of *E. granulosus and T. hydatigena* in the present study was G1. The sequences of the two isolates differed in one nucleotide. Accession numbers of two isolates of *E. granulosus* (sheep and cattle) and two isolates of *T. hydatigena* (sheep) used in this study were recorded in Gen Bank as follows: MN478490**,** MN480298**,** MN478491, MN480299.

Phylogenetic Analysis

Phylogenetic Trees with Two Methods Neighbor-Joining and Maximum Likelihood-Based on Nucleic Acid and Amino Acid for *E. granulosus*

In the plotted phylogenetic trees, the host, country, accession number, and genotype (if registered in the gene bank) are listed in each strain, respectively. Our samples are marked with a red label and out-group with a black label. Phylogenetic trees were plotted with two methods for *E. granulosus*: (neighbor-joining and maximum likelihood based on nucleic acid and amino acid). MN478490 is based on both the nucleotide sequence and the amino acid sequence in the phylogenetic tree next to the G1 genotype, which was similar to neighbor-joining and maximum likelihood, confirming that the MN478490 genotype was similar to G1. MN480298 was found in the phylogenetic tree based on nucleic acid by the neighbor-joining method next to G1, but the genotypes of the strains adjacent to those strains were not registered in the gene bank, which may not be able to judge. The phylogenetic trees concluded that the host type does not play a significant role in determining genetic affinity and the organism itself and its genotype play a decisive role in this regard. The *COX1* gene has been studied in both *E. granulosus* and *T. hydatigena*, which showed that the target gene in *T. hydatigena* was considered as an outgroup. The phylogeny based on the nucleic acid model was quite similar to both the maximum likelihood and neighbor-joining methods, confirming the validity of the plotted trees. Although the two samples in nucleic acid-based clothes have the same common ancestor together, differences in their nucleic acid composition led to sampling MN480298 in a separate clad. Based on the pairwise comparison, the two sequences were different in one nucleotide and were similar 99.77% (Figs. S4 and S5). Pairwise comparison in Fig. S6 showed that the three sequences MH010310, KX874711, and EU178104 in the same cluster with the MN480298 sequence have 100% similarity. In the case of trees based on the amino acid model, both maximum likelihood and neighbor-joining methods provided the same filtration and confirmed each other, although compared to the nucleic acid-based trees, the two samples were diverged (MN478490, MN480298 *E. granulosus*). The isolates of *E. granulosus* were different from each other in one nucleotide according to the alignment. This result indicated that the changing of nucleotide was in a non-synonymous type and that the amino acid variant caused the two studied samples to separate (Fig. S7).

Phylogenetic Trees with Two Methods: Neighbor-Joining and Maximum Likelihood Based on Nucleic Acid and Amino Acid for *T. hydatigena*

The phylogenetic trees according to the nucleic acid model were quite similar in two samples (*T. hydatigena* MN478491, MN480299) by both the maximum likelihood and neighbor-joining methods which confirms the accuracy of the drawn trees. However, the two samples we examined have the same common ancestor on nucleic acid with both methods. But, the difference in their nucleic acid composition caused the MN478491 sample was placed in a separate cloud. Based on the pairwise comparison, the two sequences were different in two nucleotides and were similar 99.55% (Figs. S8 and S9). In both methods, the phylogenetic amino acid trees had the same phylogeny (maximum likelihood and neighbor-joining) and verified the accuracy of each other. However, unlike nucleic acid-based phylogenetic trees, the two samples we examined were adjacent to one another. The isolates of *T. hydatigena* were different from each other in two nucleotides. These findings showed that the nucleotide shift was not able to modify the amino acid (Figs. S10 and S11).

Discussion

Cysticercosis caused by *T. hydatigena* and cystic echinococcosis due to *E. granulosus* causes incredible damage in the production of livestock in endemic countries [21, 29, 30]. *COX1* gene was comparable to estimate from Iranian *T. hydatigena* and *E. granulosus* populations from livestock [31, 32]. Understanding the genetic identification and characterization of the parasite will be crucial for the prevention and control of parasitic infections [8]. Mitochondrial DNA (mtDNA) sequence data have been used to study the intraspecific variation of *E. granulosus* and *T. hydatigena*. *MtDNA* is widely used in molecular and phylogenetic analysis studies [33–35]. The *COX1* gene is the most common *mtDNA* gene for phylogenetic studies, inter- and intraspecific variation, and evolutionary biology of helminth parasites [8, 33–35]. In the present study, obtained *COX1* gene subunit (455 bp) was compared with the sequences in the Gene bank. The prevalence of *E. granulosus* and *T. hydatigena* in Iranian dogs based on phylogenetic and sequence analysis of *COX1* gene *and SSU-rDNA* has shown low genetic diversity in genotypes *G3*, *G1*, and *G7* in *E. granulosus* and *T. hydatigena* [35]. In addition, using *the COX1* gene, the predominant genotypes of *E. granulosus* in humans, in Iran, were *G1, G2,*

G3, and *G6,* respectively [33, 36]. Previous studies in Iran confirm our results that *the G1* genotype of *E. granulosus* is the most common in sheep [11, 36]. In contrast to Karimi and Diantapour who reported that *the G6* genotype was the only strain present in the livestock like sheep and goats [37]. In China and Argentina, *G1* strain *E. granulosus* has been common in humans and sheep, in Nepal, *G1*, *G5*, *G6* strains among the isolates of buffalo, sheep, goats, and humans have been reported, in Eastern Europe, *G1*, *G2*, *G3*, *G6* haplotypes were reported to be the most common type of sheep [38–40]. Our results showed that the *G1* genotype of *T. hydatigena* is the most common genotype in the livestock. According to various studies performed on *T. hydatigena* based on, *nad1*, *nad5* and *COX1* gene variability, the prevalence of *T. hydatigena* in Pakistanian sheep and goats was less than 5% [22]. The first report genetic diversity of *T. hydatigena* in Sudanese sheep in isolates [23] and genetic differences in isolates from Nigerian goats and sheep [25] was reported. Also, the analysis of molecular variance *T. hydatigena* in different regions of Iran shows the molecular diversity of *12 s rRNA* in parasite isolates in goats and sheep [41]. Studies have also aimed to isolate *T. hydatigena* for the first time from liver capsules from two wild boars in Poland. This study was based on morphological indices and *COX1* sequences, which was similar to the present study in terms of sample size and detection method [26]. To complete the discussion, it should be noted that the main limitation of the present study, as in the Filip study in 2019, was the small sample size due to the rarity of the samples [26].

Conclusion

Our findings suggest that the dominant genotype in all isolates was G1 and this report was similar to other studies in Iran and the world. In addition, the partial *COX1* gene sequence was matched with *T. hydatigena*. This study opens up some interesting ideas for further research. Ideas such as: investigating the prevalence of *T. hydatigena* and *E. granulosus* based on *COX1* and *nad 1* on various animals and conducting molecular studies with higher sample sizes and comparing different genotypes of these parasites in the region.

Supplementary Information The online version containssupplementary material available at https://doi.org/10.1007/s00284-021-02377-0.

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Author Contributions VR, AI, SE helped in study concept and design; NS, OR acquired the data; SE; MS, ASK analyzed and interpreted the data; VR and SE drafted and revised the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors and coauthors declare that they have no conflict of interest that affects this study.

Ethical Approval All the studied samples have been used in this study based on ethical considerations determined by the relevant authorities (Veterinary Organization of Iran). Also, this study was the research project of Ms. Soodabeh Etemadi in the PhD course.

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