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RESEARCH ARTICLE

Molecular Characterization of Sheep Isolates of *Echinococcus granulosus* in Kilis Province

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Summary

Cystic echinococcosis is one of the most important helminthozoonosis both in Turkey and all around world. Polymerase Chain Reaction (PCR) amplification of mitochondrial 12S rRNA (mt-12S rRNA) and small subunit ribosomal RNA (rrnS) genes were used for molecular characterization of 28 isolates of *Echinococcus granulosus* obtained from 19 sheep in Kilis province. And also partial mitochondrial cytochrome c oxidase subunit I (mt-CO1) genes of randomly selected two sheep isolates were amplified and sequenced. At the end of the study, all sheep isolates were detected as *E. granulosus* sensu stricto (G1-G3 complex).

Keywords: *Echinococcus granulosus, Kilis, Sheep, PCR*

Kilis İlinde *Echinococcus granulosus*'un Koyun İzolatlarının Moleküler Karakterizasyonu

Özet

Kistik ekinokokkozis Türkiye'de ve dünyada en önemli helmintozoonozlardan biridir. Kilis ilindeki 19 koyundan elde edilen 28 *Echinococcus granulosus* izolatının moleküler karakterizasyonu için mitokondrial 12S rRNA (mt-12S rRNA) ve küçük altünite ribosomal RNA (rrnS) genleri polimeraz zincir reaksiyonu (PZR) ile çoğaltıldı. Ayrıca rastgele seçilen iki koyun izolatının mitokondrial sitokrom c oksidaz altünite 1 (mt-CO1) geni kısmi olarak çoğaltılıp dizi analizi yapıldı. Çalışma sonucunda tüm koyun izolatları *E. granulosus* sensu stricto (G1-G3 kompleks) olarak belirlendi.

Anahtar sözcükler: *Echinococcus granulosus, Kilis, Koyun, PZR*

INTRODUCTION

Cystic echinococcosis (CE) caused by the metacestode of the dog tapeworm *Echinococcus granulosus*, is a global zoonotic infection which has veterinary, medical and economic importance. The adult stages of the parasite infect canids, while the metacestodes develop in several species of wild and domestic ungulates [1,2]. Prevalence of *E. granulosus* infection in dogs varies widely between 1-54.5% in different areas of Turkey. High prevalence of CE have been reported as 4.5-81.3% in cattle, 7.2-79.6% in sheep, 1.6-32.6% in goats, 7.8% in horses and 10.24-22.3% in buffaloes [3-5]. Disease is widespread especially in eastern regions of Turkey. Prevalence rates in cattle were determined as 59.9% in Elazig, 54.1% in Malatya, 81.3% in Muş, 64.6% in Bingöl, 69.5% in Van, 57.3% in Erzincan, 43.9% in Erzurum and 43.3% in Kars provinces [5]. The numbers of surgically confirmed CE patients were 5964 between the years 1984-1986, and 21303 between 1987-1994 and the average number of new cases of hydatid diseases in Turkey is around 2000-2500 per year [6]. These data clearly show that CE is a widespread problem both in animals and humans in Turkey.

Geographically distinct strains or intraspecific variants of *E. granulosus* have been described in different intermediate host species as genotype G1 to G10. These genotypic differences affect the life cycle pattern, host

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specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, transmission dynamics, epidemiology and control of CE [1,2].

Comprehensive molecular genetic and phylogenetic analyses of Echinococcus populations have revealed that many of these strains most likely represent distinct species and reinstatement of their formal taxonomic status have been proposed [2].

According to recent taxonomic studies, *E. granulosus* was an oversimplified species, and four or five cryptic species were intermixed in it. Reviewing the names of synonyms and subspecies, the species has been split into *E. granulosus* sensu stricto (G1-G3 complex), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6-G10) [7].

Although CE continues to be an important problem in Turkey; there exist limited published information on the presence or public health impact of the different genotypes (strains, species) of *E. granulosus* [8-12]. The aim of this study was to provide molecular characterization of metacestode materials obtained from sheep in Kilis province of Turkey with Polymerase Chain Reaction (PCR) amplification of mitochondrial 12S rRNA (mt-12S rRNA) and small subunit ribosomal RNA (rrnS) and partial sequencing of the mitochondrial CO1 (mt-CO1) gene.

MATERIAL and METHODS

Collection of Cyst Material

Hydatid cyst samples were obtained from sheep at a local abattoir in Kilis province of Turkey. A total of 28 individual hydatid cyst samples from 19 sheep were examined *(Table 1)*. Four sheep had more than one cyst. All cysts were examined to determine their fertility by microscopic observation of germinal layers and protoscolices. In this study, an *E. granulosus* isolate refers to the protoscolices or cyst wall (germinal and laminar layers) obtained from a single hydatid cyst. All cyst materials were stored in 70% ethanol until the study.

Table 1. *Total number of cysts, localization and fertility*
Tablo 1. *Kistlerin toplam sayısı, yerleşimi ve fertilitesi*

Localization of Cysts	Fertile	Non-Fertile	Total
Liver	11	1	12
Lung	9	7	16
Total	20	8	28

DNA Isolation

DNA was isolated from ethanol preserved cysts. In the case of non-fertile cysts, cyst walls (germinal and/or laminar layers) were cut into small pieces. Prior to DNA extraction, ethanol-fixed protoscolices and cut cyst walls were washed five times with PBS (pH=7.4) and then digested for 6 h at 56°C within 250 µl TEN-SDS (50 mM Tris-HCl, pH8, 5 mM EDTA, 100 mM NaCl, 10% SDS) containing 2 mg/ml Proteinase-K. After digestion, 125 µl of 6M NaCl was added and the samples were vortexed for 15 s. Then the samples were centrifuged for 5 min at 13.000 g in eppendorf tubes. Finally, the supernatants were transferred to a new clean eppendorf tube, and DNA was extracted using the classical phenol-chloroform and ethanol precipitation method. After drying, the DNA was suspended in 50 µl Tris–EDTA buffer (pH=7.6) [8,13].

Polymerase Chain Reaction (PCR)

Cest4 and Cest5 primer pairs were used to amplify partial rrnS gene of *E. granulosus* regardless of strain differences [14]. While E.g.ss1for and E.g.ss1rev primer pairs were used to amplify partial mt-12S rRNA of *E. granulosus* s. s. (G1-G3 complex) [15]. And also JB3 and JB4.5 primer pairs were used for the partial mt-CO1 gene sequencing [16]. Our PCR strategy was to amplify and sequence partial mt-CO1 gene of rrnS-PCR positive and mt-12S rRNA-PCR negative isolates in order to detect strain/strains different from G1. The characteristics of primers used are summarized in *Table 2*.

PCR conditions were same for all three primer pairs. PCR was carried out in a final volume of 50 µl, containing 25.75 µl DNase, RNase free steril distilled water (Biobasic, Inc), 5 µl 10X PCR buffer, 5 µl 25 mM $MgCl_2$, 4 µl 1 mM dNTP mix, 2.5 µl of each primer (50 pmol), 0.25 µl of TaqDNA polymerase (1.25 IU) (MBI Fermentas) and 5 µl of template DNA (100-200 ng).

The PCR conditions were: 5 min at 95°C (initial denaturation), 35 cycles of 1 min at 95°C, 1 min at 50°C and 1 min at 72°C and finally 5 min at 72°C (final extension). The PCR products were separated on agarose gels (1.5%), stained with ethidium bromide, visualised and photographed on an UV transilluminator.

Table 2. *The characteristics of primers used in this study*
Tablo 2. *Bu çalışmada kullanılan primerlerin özellikleri*

Target Species	Gene	Primer Name	Sequences	Amplicon Size	Ref No
Echinococcus spp.	mt-COI	JB3 JB4.5	TTTTTTGGGCATCCTGAGGTTTAT TAAAGAAAGAACATAATGAAAATG	446 bp	16
E. granulosus	rrnS	Cest4 Cest5	GTTTTTGTGTGTTACATTAATAAGGGTG GCGGTGTGTACMTGAGCTAAAC	117 bp	14
E. granulosus (G1-G3)	mt-12S rRNA	E.g.ss1for E.g.ss1rev	GTATTTTGTAAAGTTGTTCTA CTAAATCACATCATCTTACAA T	254 bp	15

The 446 bp region of mt-CO1 gene of two liver isolates from different animals were sequenced in a trade Company (Refgen, Ankara, Turkey). The obtained sequences were edited and aligned, using the CLC sequence viewer then sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov) [17].

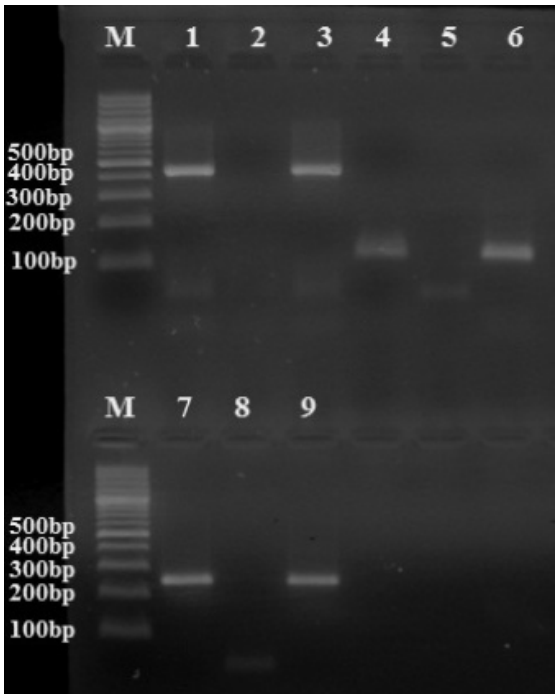

Fig 1. PCR amplification of mt-COI (1-3), rrnS (4-6) and mt-12S rRNA (7-9) genes **M:** Marker, **1,4,7:** Positive controls, **2,5,8:** Negative controls (distilled water), **3,6,9:** Sheep isolates

Şekil 1. mt-COI (1-3), rrnS (4-6) ve mt-12S rRNA (7-9) genlerinin PZR ürünleri **M:** Marker, **1,4,7:** Pozitif kontroller, **2,5,8:** Negatif kontroller (distile su), **3,6,9:** Koyun izolatları

RESULTS

The rrnS-PCR with Cest4 Cest5 primers yielded 177 bp and mt-12SrRNA-PCR with the E.g.ss1for and E.g.ss1rev primers yielded 254 bp of amplification product with all of 28 isolates analyzed. According to these results, all samples were identified as *E. granulosus* and *E. granulosus* s. s. (G1-G3 complex), respectively. The mt-CO1-PCR yielded a 446 bp-sized product in randomly selected two isolates *(Fig. 1)*. According to mt-CO1 sequences, two isolates (GenBank accession no JN810792, JN810793) were corresponding to the common sheep strain (G1). The alignment of these sequences with published sequence results (DQ062857) for sheep strain (G1) is presented in *Fig. 2*. According to the alignment results, there was 99% identity with published sequences. There was one nucleotide difference in JN810793 and two differences in JN810792 when compared with DQ062857.

DISCUSSION

To define the strains of *E. granulosus,* numerous studies have been carried out by using different molecular techniques [18]. As a result of these studies, taxonomic status of *E. granulosus* has changed day by day. Therefore, *E. granulosus* has been divided into mainly ten genotypes (G1-G10), corresponding to the strain definition and now, the species has been split into *E. granulosus* s. s. (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6-G10) [1,2,7].

In Turkey, different molecular techniques such as PCR, PCR-RFLP (restriction fragment length polymorphism), RAPD-PCR (random amplified polymorphic DNA), PZR-SSCP (single stranded conformation polymorphism) and DNA sequencing have been used to detect different strains of *E. granulosus* [8,9,19-21].

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                                20                  40                  60                  80
DQ062857 CTGGATTTGG TATAATTAGT CATATTTGTT TGAGTATTAG TGCTAATTTT GATGCGTTTG GGTTCTATGG GTTGTTGTTT 80
JN810793 .......... .......... .......... .......... .......... ....T..... .......... .......... 80
JN810792 ..........T......... .......... .......... .......... .......... .......... .......... 80

                               100                 120                 140                 160
DQ062857 GCTATGTTTT CTATAGTGTG TTTGGGTAGC AGGGTTTGGG GTCATCATAT GTTTACTGTT GGGTTGGATG TGAAGACGGC 160
JN810793 .......... .......... .......... .......... .......... .......... .......... .......... 160
JN810792 .......... .......... .......... .......... .......... .......... .......... .......... 160

                               180                 200                 220                 240
DQ062857 TGTTTTTTTT AGCTCTGTTA CTATGATTAT AGGGGTTCCT ACTGGTATAA AGGTGTTTAC TTGGTTATAT ATGTTGTTGA 240
JN810793 .......... .......... .......... .......... .......... .......... .......... .......... 240
JN810792 .......... .......... .......... .......... .......... .......... .......... .......... 240

                               260                 280                 300                 320
DQ062857 ATTCGAGTGT TAATGTTAGT GATCCGGTTT TGTGATGGGT TGTTTCTTTT ATAGTGTTGT TTACGTTTGG GGGAGTTACG 320
JN810793 .......... .......... .......... .......... .......... .......... .......... .......... 320
JN810792 .......... .......... .......... .......... .......... .......... .......... .......... 320

                               340                 360
DQ062857 GGTATAGTTT TGTCTGCTTG TGTGTTAGAT AATATTTTGC AT 362
JN810793 .......... .......... .......... .......... .. 362
JN810792 .......... .......... .......A.. .......... .. 362
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Fig 2. Nucleotide sequences of partial mt-CO1 gene for *E. granulosus* sheep strain (G1) (JN810792, JN810793) aligned with the published (DQ062857) mt-CO1 sequence of the *E. granulosus* sheep strain (G1) as a reference. A dot indicates a nucleotide that is conserved relative to the published sequence

Şekil 2. *E. granulosus* koyun suş (G1)'unun kısmi mt-CO1 geninin nükleotid dizileri (JN810792, JN810793) ile *E. granulosus* koyun suşu (G1)'nun referans (DQ062857) mt-CO1 nükleotid dizilerinin karşılaştırılması. Her bir nokta referans sekansla aynı olan nükleotidleri göstermektedir

Utuk et al.[8] used PCR-RFLP analysis of ribosomal ITS1 fragment using four different restriction enzymes (*CfoI, MspI, RsaI, AluI*) and partial sequencing of mt-CO1 gene to investigate the genetic characteristics of *E. granulosus* isolates, obtained from different hosts (179 sheep, 19 cattle, 7 goat, 1 camel, 1 dog and 1 human) and regions (Elazig, Malatya, Erzurum, Van, Diyarbakir and Sanliurfa) of Turkey. They detected common sheep strain (G1) in all examined samples.

Utuk and Piskin [22] reported sheep strain (G1) infection in a cross breed mountain goat in Antalya province by partial sequencing of mt-CO1 gene. Same researchers [19] detected *E. granulosus* s.s. (G1-G3) infection in a three month old lamb in Kıbrısçık county of Bolu province by PCR amplifying of mt-12S rRNA gene.

Simsek et al.[23] examined 220 hydatid cysts from cattle by PCR of 12S rRNA gene and sequencing of partial mt-CO1 gene. They detected *E. granulosus* s.s. (G1-G3) in 147 of 220 isolates with 12S rRNA-PCR and confirmed G1 strain by sequencing of mt-CO1 genes of 28 cattle isolates. And also they detected buffalo (G3) strain in seven cattle isolate. Simsek and Eroksuz [24] detected sheep strain (G1) infection in a Turkish mouflon (*Ovis gmelinii anatolica*) in Malatya province by partial sequencing of mt-CO1 gene.

Beyhan and Umur [4] examined nine water buffalo isolates by mt-CO1 sequencing and they detected G1 strain in six isolate while 3 samples showed variant genotypes of *E. granulosus* s. s. (G1-G3).

Simsek et al.[10] examined 70 human isolates of *E. granulosus* by 12S rRNA-PCR and mt-CO1 sequencing and they detected G1, G3 and G6 genotypes in humans. Ergin et al.[11] studied on 46 human isolates and they detected G1 strain by partial mt-CO1 sequencing. Šnábel et al.[12] examined 12 sheep and ten human isolates by DNA sequencing of four mitochondrial genes (cox1, atp6, nad1, rrnS) from western Turkey and they detected G1, G3, G1/G3 and G7 genotypes.

Studies on *E. granulosus* isolates from animals and humans have revealed that the predominant strain is G1 both in humans and in animals, and also G1/G3, G3, G6, G7 strains exist in Turkey [4,8-12,19-24].

In this study, we use two different gene regions (rrnS and mt-12S rRNA) as a target of PCR and partial mt-CO1 gene for sequencing. All examined 28 isolates were *E. granulosus* s. s. (G1-G3). In concordance with the other studies [4,8-12,19-24], *E. granulosus* s. s. (G1-G3) was predominant in sheep in Kilis province of Turkey. This is the first study about molecular characterization of sheep isolates of *E. granulosus* in Kilis province. Further molecular studies are necessary for defining the strains of *E. granulosus* and a clear understanding of the epidemiology and control of the disease.

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