

Molecular Characterization of Human and Animal Isolates of *Echinococcus granulosus* in the Thrace Region, Turkey

Canan Eryıldız¹, Nermin Şakru²

¹Clinic of Medical Microbiology, Tokat State Hospital, Tokat, Turkey

²Department of Medical Microbiology, Faculty of Medicine, Trakya University, Edirne, Turkey

ABSTRACT

Objective: *Echinococcus granulosus* is the causative agent of cystic echinococcosis in humans and many domestic animals, and remains an important global health problem. The aim of this study was to genotype *E. granulosus* isolates obtained from humans and animals in the Thrace Region of Turkey.

Material and Methods: A total of 58 isolates were obtained from patients who underwent surgery at several hospitals and from animals at a slaughterhouse in the province of Edirne. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of ribosomal internal transcribed spacer 1 fragments, and polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) of the partial mitochondrial *NADH dehydrogenase* subunit 1 (ND1) gene, was used to characterize human and animal *E. granulosus* isolates. To investigate the genetic characteristics of isolates, deoxyribonucleic acid (DNA) sequencing of the mitochondrial *cytochrome c oxidase* subunit 1 (CO1) and ND1 genes was performed.

Results: Fifty-eight *E. granulosus* isolates, including 42 from human, 13 from cattle and 3 from sheep were analyzed. The results indicated two distinct genotypes: the G1 (sheep strain) and G7 (pig strain) genotypes. The sheep strain was shown to be the most common genotype of *E. granulosus* affecting humans, sheep and cattle. Among the concatenated partial CO1 and ND1 sequence data, eight haplotypes of *Echinococcus* species were identified in the present study.

Conclusion: This is the first report indicating that the *E. granulosus* pig strain is present in humans in this region. We suggest that new strategies be designed for *E. granulosus* control programs in Turkey.

Key Words: *Echinococcus granulosus*, genotype, PCR-RFLP, PCR-SSCP, CO1, ND1, Edirne, Thrace region, Turkey

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Introduction

Echinococcus granulosus, a small cestode, belongs to the *Taeniidae* family. The adult cestode lives in the intestines of dogs and other carnivores (definitive hosts), while the larval form (metacestode) lives in the internal organs of ungulates and humans (intermediate hosts). The metacestode form causes cystic echinococcosis in intermediate hosts (1, 2). *E. granulosus* is globally distributed and causes public health problems in many parts of Eurasia, including Turkey, as well as in Africa, Australia and South America (3). Between 2001 and 2005, various hospitals and Health Ministry documents recorded 14,789 and 526 human cases of cystic echinococcosis in Turkey and Thrace region, respectively (4).

To date, considerable phenotypic and genetic variability has been observed within the species *E. granulosus*, and ten genotypes have been identified from different intermediate and definitive hosts. *E. granulosus* has been split into *E. granulosus sensu stricto* (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6-G10) (5, 6). This variability may be reflected in characteristics which affect the life-cycle pattern,

host specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, transmission dynamics, epidemiology, and control of echinococcosis (7). In particular, because of variations in the period of egg production in some strains (prepatent period in dogs for G1 genotype is an average of 45 days while this period for G7 genotype is 34 days), determination of genotypes is important in control programs (8-10).

A number of deoxyribonucleic acid (DNA) identification techniques have been employed to better characterize *Echinococcus* species and genotypes from different intermediate and definitive hosts and in different geographical settings (11). Although there are some molecular studies of *E. granulosus* isolates originating from different hosts in Turkey (12-18), no mitochondrial DNA sequence data exists for *Echinococcus* species from the European part of Turkey.

The aim of this study was to characterize the *E. granulosus* genotypes currently infecting humans and livestock animals in Turkey, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), polymerase chain reaction-single strand conformation polymorphism

Table 1. *Echinococcus granulosus* CO1 and ND1 sequence data from the present study, together with reference sequences from previous studies used for nucleotide sequence comparison and phylogenetic analysis (GenBank accession numbers)

Haplotype/ Genotype	Host origin (no)	Accession no (CO1)	Accession no (ND1)	References (CO1 and ND1, respectively)
Haplotype 1	Human (1), Cattle (1)	HQ717150	HQ717151	The present study
Haplotype 2	Human (1)	HQ717155	HQ717154	The present study
Haplotype 3	Human (1)	HQ717150	HQ717152	The present study
Haplotype 4	Human (1)	HQ717148	HQ717153	The present study
Haplotype 5	Human (1)	HQ717148	HQ717151	The present study
Haplotype 6	Human (5)	HQ703429	HQ717151	The present study
Haplotype 7	Sheep (1)	HQ717149	HQ717151	The present study
Haplotype 8	Cattle (1)	HQ717156	HQ717151	The present study
Genotype 1	Sheep	U50464	AJ237632	25, 26
Genotype 2	Sheep	M84662	AJ237633	24, 26
Genotype 3	Buffalo	M84663	AJ237634	24, 26
Genotype 4	Horse	M84664	AJ237635	24, 26
Genotype 5	Cattle	M84665	AJ237636	24, 26
Genotype 6	Camel	M84666	AJ237637	24, 26
Genotype 7	Pig	M84667	AJ237638	24, 26
Genotype 8	Moose	AB235848	AJ237643	6, 27
Genotype 10	Reindeer	AF525457	AF525297	28
<i>E. multilocularis</i> *	Human	M84668	AJ237639	24, 26
<i>E. multilocularis</i> **	Rodent	M84669	AJ237640	24, 26
<i>E. shiquicus</i>	Pika	AB208064	AB208064	6
<i>E. vogeli</i>	Rodent	M84670	AJ237641	24, 26
<i>E. oligarthrus</i>	Rodent	M84671	AJ237642	24, 26
<i>E. felidis</i>	Lion	EF558356	EF558357	29
Outgroup:				
<i>T. saginata</i>	Cattle	AB465239	AJ239106	30, 31

CO1: *Cytochrome c oxidase subunit 1*, ND1: *NADH dehydrogenase subunit 1*, *: Asterisks relate also to Figure 2

(PCR-SSCP) analyses, and to estimate the genetic variability within the strains by sequencing the mitochondrial *cytochrome c oxidase subunit 1* (CO1) and *NADH dehydrogenase subunit 1* (ND1) genes.

Material and Methods

Parasites and isolation of genomic DNA

A total of 58 isolates were obtained from patients who underwent surgery at several hospitals and from animals in a slaughterhouse all located in Edirne, in the Thrace region of Turkey, between January and December 2009. Hydatid fluid and/or germinal layers were examined by light microscopy. Also, all isolates were examined pathologically. Then, parasite materials were stored in 70% ethanol at -20°C until used. Total genomic DNA was extracted from the parasite materials using DNeasy Blood & Tissue Kit (Qiagen, Germany), according to the manufacturer's protocol.

PCR-RFLP analysis

The PCR-RFLP method was performed according to the Bowles and McManus (19) protocol with slight modifications. Ribosomal DNA-internal transcribed spacer gene 1 (rDNA-ITS1) was amplified from each sample using the BD1 and 4S primers. For each set of PCRs, positive (EU503084) (20) and negative (no-DNA) controls were included. All PCR products were digested by four-base cutting restriction endonucleases (*AluI* and *RsaI*, Bioron, Germany; *MspI* and *HhaI*, Fermentas, Lithuania). The restriction fragments were separated by electrophoresis on a 3% Tris-Borate-EDTA (TBE) agarose gel containing ethidium bromide.

PCR-SSCP analysis

Echinococcus granulosus genomic DNA samples were analyzed by PCR-SSCP using the described methods with minor modifications (21). The mitochondrial ND1 region was amplified by PCR using MS1 and MS2 primers (22). For each set

Table 2. Characteristics of *Echinococcus granulosus* human (n=42), sheep (n=3), and cattle (n=13) isolates from the Thrace region, Turkey, 2009

Host/no	Pattern of PCR-RFLP	Pattern of PCR-SSCP	DNA Sequencing		Final Genotype/ Haplotype
			CO1	ND1	
Human(1) Cattle (1)	I	A	G1	G1 (195 C/T)	G1/H1
Human (1)	III	B	G7	G7 (3 A/G)	G7/H2
Human (1)	I	C	G1	G1 (195 C/T, 288 T/C)	G1/H3
Human (1)	I	A	G1 (56 C/T)	G1	G1/H4
Human (1)	I	A	G1 (56 C/T)	G1 (195 C/T)	G1/H5
Human (5)	II	A	G1 (108 T/C)	G1 (195 C/T)	G1/H6
Sheep (1)	I	A	G1 (66 C/T, 306 G/A)	G1 (195 C/T)	G1/H7
Cattle (1)	I	A	G1 (66 C/T)	G1 (195 C/T)	G1/H8
Human (27) Sheep (2) Cattle (11)	I	A	ND	ND	G1
Human (5)	II	A	ND	ND	G1
All Isolates	I: 47 II: 10 III: 1	A: 56 B: 1 C: 1			G1: 57 G7: 1

PCR-RFLP: Polymerase chain reaction - restriction fragment length polymorphism, PCR-SSCP: Polymerase chain reaction - single stranded conformation polymorphism, CO1: *Cytochrome c oxidase* subunit 1, ND1: *NADH dehydrogenase* subunit 1, G: Genotype, C:Cytosine, T: Thymine, G: Guanine, A: Adenine, H: Haplotype, ND: Not Done

of PCRs, positive [G1: EU503084 (20), and G6-G10 complex: GU951511 (18)] and negative (no-DNA) controls were included. Four microliters of each PCR products were mixed with 12 µl of denaturing buffer. After denaturation at 95°C for 10 min and subsequent snap-cooling on a freeze block, 10 µl of individual samples were loaded into the wells. Electrophoresis was carried out on 12% acrylamide-bisacrylamide non-denaturing gels containing 5% glycerol at 200 V for 20 hours at room temperature. The gel was stained using the silver-staining technique modified by previously described methods (23).

Mitochondrial DNA sequencing and phylogenetic analysis

Thirteen amplicons, representing each unique SSCP and RFLP profile, were selected, and fragments of amplicons mitochondrial CO1 and ND1 genes were amplified with primers published in Bowles et al. (24) and Sharbatkhori et al. (22), respectively. Then, PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Germany). Sequencing was performed on the ABI 3130 XL Genetic Analyzer (Applied Biosystems, USA) with the BigDye Cycle Sequencing Kit (Applied Biosystems, USA) using the corresponding PCR primers by Refgen Company (Ankara, Turkey). DNA sequences were compared with partial CO1 and ND1 sequences (6, 24-31) representing all currently known genotypes of *E. granulosus*, all species of *Echinococcus* and *Taenia saginata* (the out-group) (Table 1) which we obtained from previous publications and National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>). Phylogenetic analysis of partial sequence data from mitochondrial CO1 and ND1 genes was

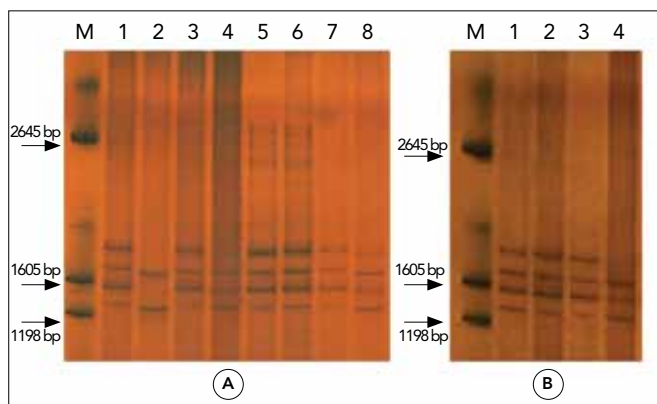


Figure 1. SSCP analysis of mitochondrial ND1 PCR products of *Echinococcus granulosus*

M: marker (Promega, USA); A: G1 control (lane 1), G6-G10 complex control (lane 2), pattern A (lane 3,4,5,6 and 7) and pattern B (lane 8); B: G1 control (lane 1), pattern A (lane 2 and 3), pattern C (lane 4)

performed by Iontek Company (İstanbul, Turkey). Phylogenetic analysis of concatenated CO1 and ND1 data was conducted by the maximum likelihood (ML) method, employing the MEGA5 (Molecular Evolutionary Genetics Analysis) software.

Results

PCR-RFLP analysis

ITS1-PCR was carried out on 58 isolates from 42 humans, 13 cattle and 3 sheep. All isolates showed two fragments of

approximately 1000 bp and 1100 bp. ITS-PCR products of the 58 isolates were digested by restriction endonucleases, and band patterns were compared with the band patterns of reference isolates. The results demonstrated that 47 isolates had the same band pattern as the reference G1 isolate (pattern I), 10 isolates had pattern II and one isolate had pattern III (Table 2).

PCR-SSCP analysis

ND1-PCR was carried out on 58 isolates, and all isolates showed a fragment of approximately 400 bp. SSCP analyses of all 58 amplicons displayed three unique profiles for partial ND1. These patterns were compared with the domestic sheep strain and *E. canadensis* patterns. 56 isolates (pattern A) corresponded to the G1 strain pattern. Two human isolates (patterns B and C) produced different patterns from the reference G1 and G6-G10 complex strains (Figure 1, Table 2).

Mitochondrial DNA sequencing and phylogenetic analysis

Thirteen amplicons, representing each unique RFLP and SSCP profile, were selected. ND1 and CO1-PCR were carried out on these isolates, and all isolates showed fragments of approximately 400 bp and 450 bp, respectively. Twelve isolates, including nine human, two cattle and one sheep were determined to be the G1 strain, while one human isolate was determined to be the G7 strain. The obtained sequences were submitted to the GenBank database under their accession numbers (Table 1).

The CO1 region of three of the 13 isolates fully corresponded with the G1 reference sequence. Two isolates possessed the non-synonymous nucleotide substitution 56 C/T (inducing substitution of alanine with valine). One isolate presented a C/T exchange at position 66. Five human isolates possessed the nucleotide substitution 108 T/C. One sheep isolate presented two mutations (66 C/T and 306 G/A). One human isolate produced a sequence which fully corresponded with the G7 reference sequence.

The ND1 region of one of 13 isolates fully corresponded with the G1 reference sequence. Ten isolates possessed the nucleotide substitution 195 C/T. One isolate presented two mutations (195 C/T and 288 T/C). One human isolate, which was identified as the G7 genotype, had a guanine nucleotide at position 3. All of these nucleotide substitutions were synonymous.

Among the concatenated partial CO1 and ND1 sequence data, eight haplotypes were detected in the present study using reference data (comprising concatenated partial sequences from previous studies representing all recognized *E. granulosus* genotypes; Table 1). Based on the phylogenetic analyses, haplotype 2 were classified as G6-G10 complex; and the other seven haplotypes (haplotype 1, 3-8) were classified as G1-G3 complex (Figure 2).

Discussion

Some studies investigating the molecular characterization of *E. granulosus* isolates from humans, sheep, goats, cattle, camels and dogs in different parts of Turkey have been performed previously. The investigators used different methods

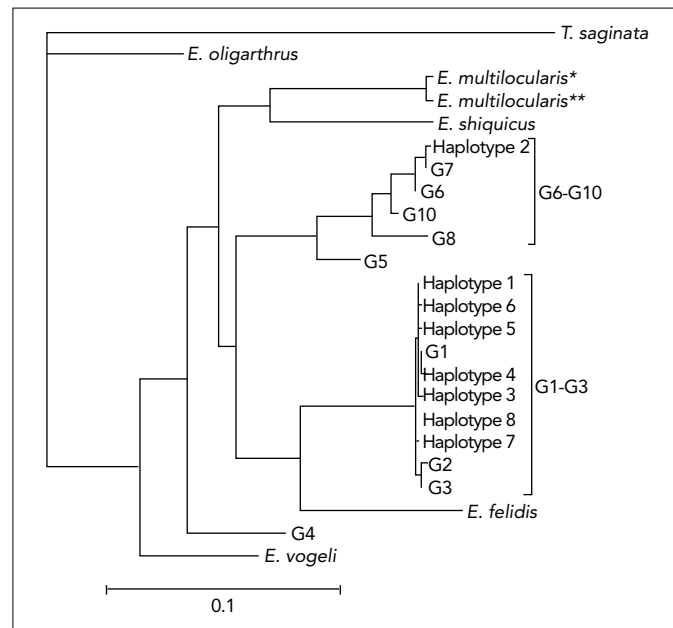


Figure 2. Genetic relationships of haplotypes 1-8, which represent *Echinococcus granulosus* samples from Turkey (present study), to reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analysis of concatenated CO1 + ND1 sequence data (see Table 1) using maximum likelihood (ML) method

to classify *E. granulosus* isolates from different hosts in Turkey, such as PCR-RFLP of the ITS-1 region of the rRNA gene and sequencing of CO1 (15); comparative sequencing of the CO1 and/or ND1 genes (12-14); specific PCR analysis of the 12S rRNA gene and sequencing of CO1 (16, 18); and specific PCR analysis of the 12S rRNA gene. Another study performed SSCP analysis of the CO1 gene and sequencing of CO1 (17). According to these reports, G1 was the most prevalent strain among human and animal isolates. The G1 genotype has been demonstrated in humans, sheep, goats, cattle, camels, water buffaloes and dogs (12-16); the G3 genotype has been demonstrated in humans, sheep and cattle (12, 13); and the G7 genotype has been demonstrated in humans and sheep (13), whereas G6 has only been identified in humans (18).

The present study showed that G1 is the dominant isolate in Turkey and is the principal agent in human and animal cystic echinococcosis. In addition, the G7 genotype was detected for the second time in human isolates from Turkey. In the previous study, the pig strain G7 was reported in one human isolate and two isolates from sheep (13). Our results are consistent with the data obtained by other authors who used different techniques, such as PCR-RFLP (15, 32-37) and SSCP (17, 22, 38-40) analyses for the genotyping of *E. granulosus*. In addition, we examined 13 isolates of *E. granulosus* from different intermediate hosts using sequence analysis of the mitochondrial ND1 and CO1 genes. We identified two *E. granulosus* genotypes (G1 and G7). Although the human, sheep and cattle isolates shared identical sequences to the sheep strain (G1 genotype), one human isolate produced identical sequences to the pig strain (G7 genotype). Previous analyses (22, 39, 40) revealed that concatenated sequence

data was reliable for estimating haplotypic variability within *E. granulosus*. The present phylogenetic analysis classified 12 isolates (representing haplotype 1, 3-8) as *E. granulosus sensu stricto* (G1-G3 complex) and one isolate as *E. canadensis* (G6-G10 complex).

In the present study, analysis of partial mitochondrial CO1 sequence data revealed a C/T change at position 56 (HQ717148), the only mutation in our study that causes an amino acid change. Similar C/T changes were seen in many sequences including Turkey isolates, such as EU178104, JF775379, JF775380, HM598459, EF689726, AB491447 and AJ508019 deposited in the GenBank database (12-15, 41, 42). The C/T change at position 66, which was identified in a cattle isolate (HQ717156), has been previously noted in various parts of the world (HM130578, AB491456, DQ356881) (41, 43, 44). Similar patterns recorded in the study of Vural et al. (12) from eastern Turkey were classified as G3 (EF545563, EU178105) and Snabel et al. (13) from western Turkey were classified as G1/G3 genotype cluster.

The T/C change at position 108, which was identified in our human isolates (HQ703429), was reported in the sheep isolate (AJ508005) in Australia (42). The sequence in which the 66C/T and 306G/A changes existed together was identified in the sheep isolate (HQ717149) and was not found among the other sequences in GenBank. However, a human isolate (AJ508024) with a 306G/A mutation was identified by Obwaller et al. (42).

Analysis of partial mitochondrial ND1 sequence data revealed a C/T change at position 195 in human, sheep and cattle isolates (HQ717151) that has been reported in many isolates from various parts of the world (including HM853645, DQ856470 and AF408688, among others) and from Turkey (13, 45-47). The co-occurrence of the 195 C/T and 288 T/C changes (HQ717152) has been previously noted [FJ796212 (22)]. In our study, the human isolate identified as the G7 genotype had a guanine nucleotide at position 3 (HQ717154), unlike the reference G7 sequence. The 3A/G mutation was not found in the sequences deposited in the GenBank database.

The domestic sheep strain (G1) of *E. granulosus* has a worldwide geographical distribution. It is found in parts of South America, South and East Europe, North and East Africa, Asia and Australia. The pig strain (G7) is found in some countries in central and east Europe, parts of Russia, and Mexico and Peru (48-50). The pig strain is considered to have low infectivity for human, however, infected human cases with pig strain have been recorded in Poland and Turkey (8, 13, 51).

A study including sheep and goats in Greece, Turkey's neighbour which has been placed in the North Thracian region, showed G1 and G3 genotypes in sheep, and G7 genotype in all goats (46). In Bulgaria, another neighbour, Breyer et al. (52) detected G1 genotype in all *E. granulosus* isolates including cattle, sheep, pigs, jackals and a wolf. Casulli et al. (53) investigated the mitochondrial variability of *E. granulosus* s.s. in the European countries of Bulgaria, Hungary, Romania and Italy. Some isolates were identified as G7 genotype from Hungary and Romania. All other isolates were identified as *E. granulosus sensu stricto*.

There is a single pig farm in Turkey, however, because the majority of Turkey's population are Muslims, and pork con-

sumption is very low. In our study, the single human case infected with pig strain has never left the Thrace region. Although there is no pig farm in the Thrace region, the number of wild boars is not low. For these reasons, we suppose that the pig strain obtained from a patient from Edirne may have come through a wild cycle from the neighbouring countries. However, in order to speak more clearly about this subject, studies which include more patients, livestock, wild boars and definitive hosts from a wide geographical area are required.

Conclusion

Our genotypic data strongly suggest that most of the human and animal isolates are of the G1 genotype. As this study identifies the G7 genotype in human isolates from the Thrace region for the first time, and because the G7 and G1 genotypes have different maturation periods in definitive hosts, we suggest that new strategies be designed for further genetic analysis and *E. granulosus* control programs in Turkey.

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Conflict of Interest

No conflict of interest was declared by the authors.

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