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ORIGINAL PAPER

A comprehensive molecular survey of *Echinococcus granulosus* in formalin-fixed paraffin-embedded tissues in human isolates in Turkey

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Abstract Cystic echinococcosis (CE) due to the formation of a hydatic cyst is a disease commonly seen in humans and animals that can be mortal sometimes. This disease, which is present in many countries around the world, constitutes a great problem for public health and the economy. The aim of this study was to identify *Echinococcus granulosus* genotypes in formalin-fixed, paraffin-embedded tissues. Tissue samples from 70 human patients with histologically confirmed echinococcosis were analysed by direct PCR of the 12S rRNA gene and by DNA sequence analysis of the *CO1* gene of *E. granulosus*. Of the 70 samples, 29 (41.6%) could be genotypically characterized. Specifically, 26 of 70 were positive by direct G1-3 PCR of the 12S rRNA gene, two of which were identified as G1 by additional *CO1* gene sequencing. All the 44 unidentified samples underwent *CO1* sequencing, which yielded one G3 and two G6 records, while the remaining 41 samples gave no or inconclusive results. In conclusion, the results from the analysis of human isolates of *E. granulosus* confirmed the occurrence of G1, G3 and G6 genotypes in Turkey and indicated G1/G3 cluster (*E. granulosus* sensu stricto) as the predominant genotype.

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Introduction

Cystic echinococcosis (CE) is a zoonotic infection caused by the larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* and the family Taeniidae (Thompson 1995). The parasites are perpetuated in life cycles with carnivores as definitive hosts, which harbour the adult egg-producing stage in the intestine, and intermediate host animals, in which the infective metacestode stage develops after peroral infection with eggs. Metacestodes may incidentally also develop in humans, causing various forms of echinococcosis, and this may also occur in various animal species, which do not play a role in the developmental cycle of the parasite. A number of intraspecific variants or strains are known to occur within the species *E. granulosus*. The term ‘strain’ is used to describe variants which differ statistically from other groups of the same species in gene frequencies and in one or more characters of actual or potential significance to the epidemiology and control of echinococcosis. This variability may be reflected in characters which affect the life-cycle pattern, host specificity, development rate, pathogenicity, antigenicity and sensitivity to chemotherapeutic agents, transmission dynamics, epidemiology and control of echinococcosis (Thompson and Lymbery 1990). However, some of the forms which have been recognized as distinct strains were, in fact, described many years ago as species or subspecies. The reinstatement of their formal taxonomic status has recently been proposed following a reappraisal of the taxonomy of *Echinococcus* in light of phylogenetic analyses of DNA sequence data (Thompson et al. 1995). *E. granulosus* comprises a number of genetic variants. To date, molecular analysis, mostly using mitochondrial DNA (mtDNA) sequences, has indicated ten distinct genotypes (G1–G10) or strains within *E. granulosus* (Bowles et al.

1992; Thompson 1995). Recently, the G4 (horse strain) and G5 (cattle strain) genotypes have been suggested as distinct species, namely *Echinococcus equinus* and *Echinococcus ortleppi*, respectively (Nakao et al. 2007). In addition, there are some controversies for considering G6 to G10 as one or two species, namely *Echinococcus canadensis* and *Echinococcus intermedius*. The validity of the G9 genotype has been questioned (Nakao et al. 2010; Saarma et al. 2009). Thus, the taxonomy of *E. granulosus* is not precisely clear (Sharbatkhori et al. 2010).

Until now, limited reports on the strain characteristics of *E. granulosus* in Turkey have been published. Bowles et al. (1992) analysed a single sheep isolate sent from Turkey and found it to be the G1 genotype, a similar finding to that obtained by Obwaller et al. (2004). Also, Schneider et al. (2008) established a new PCR protocol for the detection and discrimination of *E. granulosus* complex in formalin-fixed, paraffin-embedded (FFPE) tissues, examined 20 patients of Turkish origin and detected only the G1 strain. Utuk et al. (2008), examined 179 sheep, 19 cattle, 7 goat, 1 camel, 1 dog and 1 human isolate by using PCR-RFLP of the ribosomal ITS1 gene region and mitochondrial *CO1* sequence analysis and determined only the sheep strain (G1) in all samples. Vural et al. (2008) investigated 100 sheep and 12 cattle isolates of *E. granulosus* derived from different parts of Turkey by mt-*CO1* gene sequence analysis. They (Vural et al. 2008) reported that haplotypes were identified, which corresponded clearly to the previously described strain G1 in a total of 107 isolates, including 98 isolates from sheep and nine isolates from cattle. Five isolates, including two sheep and three cattle, were determined to belong to the G3 genotype. Snabel et al. 2009 analysed 12 sheep and 10 human isolates by DNA sequencing of four mitochondrial genes. They detected the G1 and G3 genotypes besides the G1/G3 cluster. The authors also reported the pig strain (G7 genotype) in three isolates from sheep and humans for the first time in Turkey. Simsek and Eroksuz (2009) described the first genotypic analysis of *E. granulosus* infecting Turkish mouflon by using DNA sequencing in Turkey and found the G1 genotype. In a study conducted by Simsek et al. (2010), 220 cattle hydatid cyst samples were analysed, and only G1 and G3 genotypes were detected. Lastly, Ergin et al. (2010) sequenced the mt-*CO1* gene regions of 46 hydatid cyst samples collected from humans during surgery and found only the G1 genotype by using DNA sequence analysis in all samples.

G1 and G3 strains of *E. granulosus* are most commonly associated with both human and animal infections worldwide and in Turkey as well. Whereas camels are commonly infected in the Middle East and Africa, opinions have differed regarding the infectivity of *E. granulosus* of camel origin to humans. Dinkel et al. (2004) analysed 25 goat isolates by specific PCR and found all of them to be G6/G7 strains, of which seven were detected to be the G6 strain by confirmatory DNA sequencing analysis of *CO1* and *nad1* genes. This shows that the G6 strain infects camels and goats as well. Since the G6 genotype had been identified as the aetiological agent only in sporadic cases of CE, it was believed that this strain was less infective for humans or not infective at all. In 1999, the G6 genotype was identified in humans for the first time, in Argentina (Rozenzvit et al. 1999). Cases of human infection with the G6 genotype were later reported in South America (Argentina, Chile and Peru), Asia (China, Iran and Nepal) and Africa (Egypt, Mauritania, Sudan and Kenya) (Casulli et al. 2010). These findings indicate that the G6 genotype has a wider distribution than previously thought.

The objective of the present study was to identify which *E. granulosus* genotypes are the causative agents of CE in humans by using molecular analysis in FFPE tissues as a source of DNA.

Materials and methods

Isolates

Tissues samples from 70 human patients with histologically confirmed echinococcosis, operated on between 2000 and 2009 at the University Hospital in the Elazig province of eastern Turkey, were investigated in this study. All patients were identified as being infected with CE by histopathological examination (detection of PAS-positive laminated layers and/or protoscoleces and/or hooklets) of the resected tissues. Thirty six of the patients were female; 27 were male, and the remaining seven were not determined. The age range was 4–74 years (average, 32 years).

Molecular analysis

For the extraction of genomic DNA (gDNA), 10-μm thick sections were prepared from tissue blocks, and excess paraffin was trimmed. Disposable razor blades were used, and all equipment were cleaned carefully to prevent possible contamination between tissues. Sections were placed in Eppendorf tubes and deparaffinized with 1 ml xylene for 10 min at 37°C. Subsequently, samples were centrifuged at 5,000 rpm for 5 min, and the supernatant was removed. This procedure was repeated once. After deparaffinization, rehydration in 100%, 90%, 80% and 70% ethanol followed (Schneider et al. 2008). Thereafter, the 70% ethanol was removed, and a commercial kit of lysis buffer was added. Total gDNA was extracted from each individual sample using a commercial tissue DNA isolation kit (Genomic DNA Purification Kit-K0512, Fermentas)

with few modifications. The tissues were digested overnight at 56°C within 400 µl of the lysis buffer with the addition of 40 µl Proteinase-K (20 mg/ml) (Sigma, USA). After digestion, the gDNA extraction kit procedure was performed. At the last step, the pellets were resuspended in 80 µl of sterile distilled water, and the gDNA samples were stored at −20°C until use.

The mitochondrial 12S rRNA gene was amplified using specific primers previously described by Dinkel et al. (2004): *E.g.*ss1for. 5′-GTATTTTGTAAAGTTGTTCTA-3′ and *E.g.*ss1rev. 5′-CTAAATCACATCATCTTACAAT-3′. PCR products were carried out in a final volume of 50 µl, containing 5 µl 10× PCR buffer, 5 µl 25 mM $MgCl_2$, 250 µM each of dNTP, 20 pmol of each primer, 200 ng of template DNA and 1.25 IU of TaqDNA polymerase (MBI Fermentas). The PCR conditions were 3 min at 95°C (initial denaturation), 40 cycles of 30 s at 94°C, 1 min at 57°C and 40 s at 72°C and finally, 5 min at 72°C (final extension). The PCR products were separated on agarose gels (1.4%) and stained with ethidium bromide.

Fragments of the *CO1* mitochondrial gene were amplified as reported by Bowles et al. (1992) using the JB3/JB4.5 primers (5-TTTTTTGGGCATCCTGAGGTTTAT-3/5-TAAAGAAAGAACATAATGAAAATG-3). PCR amplification was performed in a 50-µl volume containing gDNA (100 ng), 250 µM of each dNTP, 2.5 mM of $MgCl_2$, 20 pmol of each primer, 5 µl of 10× PCR buffer and 1.25 U of TaqDNA polymerase (MBI, Fermentas; Lithuania). The PCR conditions were: 5 min at 95°C (initial denaturation), 35 cycles of 50 s at 94°C, 50 s at 45°C and 50 s at 72°C and finally, 10 min at 72°C (final extension). The PCR products were separated on agarose gels (1.4%) and stained with ethidium bromide. Subsequently, bands were cut from the gel, and amplified DNA fragments were purified by QIAquick Gel Extraction Kit (Qiagen). The *CO1* sequences were automatically obtained using a 377 ABI PRISM system (Applied Biosystems). Nucleotide sequence analysis was undertaken by BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

Results

A total of 70 FFPE tissue samples were tested for the molecular analysis. All these samples were analysed using 12S rRNA primers. During the molecular analysis, 32 samples were analysed in duplicate. The 12S rRNA PCR with the *E.g.*ss1for. and *E.g.*ss1rev. primers yielded 254 bp of amplification product in 26 (37.2%) samples analysed. These samples were identified as G1/G3 cluster (*E. granulosus* sensu stricto) strain. The sequencing of the mitochondrial *CO1* gene of the two samples, which were randomly selected amongst these 26 samples, produced sequences of 446 bp for each sample analysed. Both of the examined *CO1* sequences were identified as corresponding to the common sheep strain (G1) of *E. granulosus* (Genbank accession numbers GU951512–13). The other 44 samples were analysed for the mitochondrial *CO1* gene, and only four samples yielded a 446-bp product. After the *CO1* sequence analyses of four isolates, one of them was identified as corresponding to the buffalo strain (G3) (Genbank accession number GU951515). This isolate was from a 59-year-old female. Finally, the isolates from the remaining two patients were surprisingly detected as the camel strain (G6) of *E. granulosus* (Genbank accession numbers HM031460 and GU951511). The alignment of these sequences with the published sequence result (AB274020) is presented in Fig. 1. One of these patients was an 8-year-old male from Bingöl province, and the other was a 74-year-old female from Tunceli. The last sample was considered to be inconclusive despite repeated sequence analyses. Age, sex, origin and genotype properties of the 29 successfully examined patients are shown in Table 1.

Discussion

Formalin has been used as a fixative in pathology for more than a hundred years. The criteria employed by pathologists for the diagnosis of many diseases have been established in FFPE tissue sections stained with haematoxylin and eosin. During more than a century of diagnostic practice, a large number of archival paraffin-embedded tissue banks have been established worldwide. These tissue banks form invaluable resources of tissues for translational studies of cancer and various other diseases (Shi et al. 2002). Numerous studies have been reported on the genetic characterization of both human and animal *Echinococcus* isolates; however, the material used for these studies have been germinal membranes, protoscoleces, and adult parasite tissues (Dinkel et al. 2004). In general, collection of fresh human cystic material is problematic, except in hospitals located in highly endemic areas for *E. granulosus*. On the other hand, paraffin blocks are easy to transport and store in the laboratory (Schneider et al. 2008).

In the present study, we amplified *E. granulosus*-specific DNA in 29 out of 70 (41.4%) FFPE tissue samples (histologically confirmed). In 41 (58.6%) samples, we were unable to detect specific DNA fragments. At least two reasons might be responsible for the negative results, with one of them being DNA degradation (Schneider et al. 2008). The paraffin blocks and slides investigated in this study were between 10 years and a few months old when received for analysis. Formalin has usually been used for

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                           20                                        40
P59 (HM031460)    TATTTGTTTG AGGATTAGTT CTAATTTGGA TGTTTTTGGG  40
P97 (GU951511)    .......... .......... .......... ..........  40
E.gran.G6 (AB274020) ..........  .......... .......... ..........  40
                           60                                        80
P59 (HM031460)    TTTTATGGGT TGTTGTTTGC TATGTTTTCT ATAGTGTGTT  80
P97 (GU951511)    .......... .......... .......... ..........  80
E.gran.G6 (AB274020) .......... .......... .......... ..........  80
                          100                                       120
P59 (HM031460)    TAGGTAGTAG TGTTTGGGGA CATCATATGT TTACTGTTGG 120
P97 (GU951511)    .......... .......... .......... .......... 120
E.gran.G6 (AB274020) .......... .......... .......... .......... 120
                          140                                       160
P59 (HM031460)    ATTAGATGTG AAGACTGCTG TTTTTTTTAG TTCTGTTACT 160
P97 (GU951511)    .......... .......... .......... .......... 160
E.gran.G6 (AB274020) .......... .......... .......... .......... 160
                          180                                       200
P59 (HM031460)    ATGATTATAG GTGTTCCTAC TGGTATAAAG GTGTTTACTT 200
P97 (GU951511)    .......... .......... .......... .......... 200
E.gran.G6 (AB274020) .......... .......... .......... .......... 200
                          220                                       240
P59 (HM031460)    GGTTGTATAT GTTATTGAAT TCTAATGTTA ATGCTAGTGA 240
P97 (GU951511)    .......... .......... .......... .......... 240
E.gran.G6 (AB274020) .......... .......... .......... .......... 240
                          260                                       280
P59 (HM031460)    TCCTGTTTTG TGGTGGGTTA TTTCTTTTAT AGTTTTATTT 280
P97 (GU951511)    .......... .......... .......... .......... 280
E.gran.G6 (AB274020) .......... .......... .......... .......... 280
                          300                                       320
P59 (HM031460)    ACGTTTGGGG GCGTCACTGG TATAGTTTTG TCTGCTTGTG 320
P97 (GU951511)    .......... .......... .......... .......... 320
E.gran.G6 (AB274020) .......... .......... .......... .......... 320
                          340                                       360
P59 (HM031460)    TGTTGGATAA TGTTTTACAT GATACTTGGT TTGTAGTAGC 360
P97 (GU951511)    .......... .......... .......... .......... 360
E.gran.G6 (AB274020) .......... .......... .......... .......... 360
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Fig. 1 Nucleotide sequences of a fragment (360 bp) of mitochondrial cytochrome *c* oxidase subunit 1 (CO1) for two isolates of *E. granulosus* analysed in the study aligned with the published (GenBank TM/EBI Data Bank accession no. AB274020) *CO1* sequence of the G6 genotype as a reference. A *dot* indicates a nucleotide that is conserved relative to the published G6 sequence

fixation of clinical tissue samples for several years, with the duration of fixation being highly variable. However, fixation time and also the choice of fixative significantly affect the quality of the extracted DNA. We could not obtain any retrospective data on the length of fixation, temperature and use of various fixatives. A total of 32 samples were analysed twice to overcome the negative results at both DNA isolation and PCR steps in this study. However, none of them yielded positive results. Another reason for the negative results might be the inhibition of PCR by the high polysaccharide content of germinal/laminar layers of cyst tissues (Schneider et al. 2008). Kamenetzky et al. (2000) described DNA extraction from the germinal membrane of non-fertile hydatid cysts followed by strain determination using mt-*CO1* gene sequencing. The polysaccharide-containing laminar layers of the non-fertile cysts were supposed to contain PCR inhibitors, and the authors described a protocol to remove those. However, the protocol recommended for the removal of inhibitors was not applicable to FFPE tissues (Schneider et

Table 1 Age, sex, origin and genotype characteristics of 29 successfully examined patients

Ages	0–15	16–30	31–45	46–60	≥60	Undetected
	8	4	9	3	3	2
Origins	Elazig	Bingöl		Tunceli		
	20	6		1		2
Sexes	Female			Male		
	13			14		2
Genotypes	G1–G3 26	G3 1		G6 2		
Total			29			

al. 2008). We used the 12S rRNA gene as a target region to detect the G1/G3 cluster and mt-*CO1* gene for the detection of other strains by sequencing (Bowles et al. 1992; Dinkel et al. 2004). We used the *CO1* gene sequence with the aim to confirm the *E. granulosus* strains as reported by others (Manterola et al. 2008; Utuk et al. 2008; Simsek and Eroksuz 2009).

The majority of our samples (n=26; 37.1%) were identified by PCR as G1/G3 cluster (*E. granulosus* sensu stricto); one was (1.4%) G3 genotype (buffalo strain), and two (2.8%) were G6 genotypes (camel strain) by DNA sequence analysis.

It was believed previously that the G6 genotype was less infective for humans or not infective at all. However, the G6 genotype was identified in humans for the first time in four of nine patients with CE in Argentina in 1999. Then, 17 human cases with the G6 genotype were reported in some other countries (Casulli et al. 2010). These findings indicate that the G6 genotype has a wider distribution than previously thought. Based on previous molecular studies, the G1 genotype of *E. granulosus* exists in Turkey (Schneider et al. 2008; Utuk et al. 2008; Vural et al. 2008; Simsek and Eroksuz 2009). In this study, three genotypes of *E. granulosus* were identified using PCR of the 12S rRNA gene and sequence analysis of mt-*CO1* gene fragments in 70 human isolates obtained from FFPE tissues from Turkey. Previous studies in Turkey indicated that the so-called sheep strain (G1) was the most prevalent genotype of *E. granulosus* in both livestock (Utuk et al. 2008; Vural et al. 2008) and humans (Schneider et al. 2008). However, in this study, the G1/G3 cluster was detected in human isolates by PCR, and besides, G1, G3 and, interestingly, G6 genotypes were found in humans by DNA sequence analysis..

The finding of G6 in humans is indicative of interactions between the camel–dog, sheep–dog, goat–dog and human–dog cycles. Although the camel population is quite lower in Turkey than those in southeast neighbours like Iran, Iraq and Syria, due to the illegal animal transport from the border, the strain transition is possible. Where camels and other livestock live together, there is a possibility of exposure to other strains through contact with the common definitive hosts, particularly dogs. This issue has important implications for the epidemiology of *E. granulosus* and should be considered in any control programmes targeting hydatid disease.

The G6–G10 cluster (*E. canadensis*) includes not only strains found in wildlife in the northern Palearctic, but also the 'domestic' camel strain (G6) of the Middle East and northern Africa and the pig strain (G7) of central–eastern and southern Europe. All are infective to humans, but reported cases are much rarer than those with G1 (Snabel et al. 2009). The authors (Snabel et al. 2009) collected 12 sheep and ten human isolates which were examined by DNA sequencing of four mitochondrial genes (*cox1*, *atp6*, *nad1*, *rrnS*). They found the G1 genotype (including three microvariants) in 17 isolates from humans and sheep, besides the G3 genotype and an intermediate form G1/G3 in one isolate each (both from sheep). Interestingly, *E. canadensis*, the pig strain G7, was reported in three isolates from sheep and humans. This was the first report of the G7 strain in Turkey. At least ten genotypically defined strains (G1–G10) were described within the *E. granulosus* complex, some of which exhibit marked biological and morphological differences. Such genotypes were recently proposed to merit species status, namely *E. granulosus* sensu stricto (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–G10) (Lavikainen et al. 2003; Thompson et al. 2006; Nakao et al. 2007; Moks et al. 2008). Thus, we can name the G6 genotype as *E. canadensis* according to the last phylogenetic taxonomy of *Echinococcus* species.

In conclusion, our results from the analysis of human isolates of *E. granulosus* confirmed the existence of G1, G3 and G6 genotypes and the high prevalence of G1/G3 cluster in humans. In addition, the G6 genotype was detected for the first time in human isolates in Turkey. Due to the potential presence of other *E. granulosus* genotypes, we suggest surveys on more *E. granulosus* isolates from humans and other intermediate hosts along with dogs as the definitive host in various geographical regions of Turkey.

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