



Evaluation of *Echinococcus* DNA by polymerase chain reaction (PCR) in cystic *Echinococcosis* of the liver

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ABSTRACT

Objective: The aim of this study was to determine the DNA and genotypes of *Echinococcus granulosus* in liver cyst hydatids isolated in humans.

Material and Methods: This study was conducted prospectively at the Department of General Surgery of the Cerrahpaşa School of Medicine, University of İstanbul-Cerrahpaşa, between January 2015 and June 2016 in 30 patients who were operated on for cystic *Echinococcosis*. *E. granulosus* DNA was analyzed using the Polymerase Chain Reaction (PCR) method in the cyst samples (protoscolex and/or germinative membrane) obtained during the operation, and genotype was determined in the PCR positive samples by sequence analysis. At the same time, indirect hemagglutination (IHA) was used to test for the presence of antibodies in the patients' blood.

Results: *E. granulosus* DNA was found in 29 out of 30 cystic *Echinococcosis* of the liver samples. All of the 29 cystic *Echinococcosis* samples were found to be G1 (sheep) species. Also, IHA was positive in 22 patients and negative in eight patients.

Conclusion: In the present study, G1 species was the most commonly seen liver cystic *Echinococcosis* species. We suggest that a vaccine, which could be developed against prevalent regional genotypes, would be efficacious in the prevention of the disease with a cause of mortality and morbidity.

Keywords: Liver cystic *Echinococcosis*, *Echinococcus granulosus*, genotype, DNA, PZR, Western-Blot

INTRODUCTION

The disease, caused by the settling of the larva form (metacestode) of *Echinococcus granulosus* in many organs, including primarily the liver and lungs in humans, is called "cystic *Echinococcosis*" (CE: Cystic *Echinococcosis*). CE is a zoonotic disease, caused by *E. granulosus*, *E. multilocularis*, *E. vogeli* and *E. oligarthrus*, which is seen most frequently in the liver, lungs and spleen, as well as in various other localizations in recurrent cases worldwide; however, mainly in countries such as Turkey, where there is widespread animal husbandry (1,2). The main hosts of *E. granulosus* are carnivores such as dogs, wolves, and foxes. Sheep, cattle and humans are intermediate hosts in the life cycle of the parasite. The intermediate host is infected through food contaminated with the eggs of the parasite. (3) Subsequently, the embryos pass to the portal blood circulation through the intestines and settle in the liver (50-70%), lungs (10-30%), spleen, kidney, central nervous system, bone and muscle tissue respectively, and develop the cyst form that is the larval period (3,4). *E. granulosus* is the one among all *Echinococci*, and 10 different genotypes of *E. granulosus* have been determined as domestic sheep species (G1), The Tasmania sheep species (G2), buffalo species (G3), [G1- G3 species have been grouped as *E. granulosus sensu stricto* (s.s)], horse species (G4), bovine species (G5), camel species (G6), pig species (G7), deer species (G8), wild life species (lion species, lagomorph species) (G9) and Fennoscandian deer species (G10) (5,6). *Echinococcosis* is seen worldwide and most frequently in Eurasia, the Mediterranean, North and East Africa and South America (2,6,7). Genetic diversity has a critical significance in the determination of the phenotypical properties, host specificity, efficacy of spread, pathological processes, antigenicity, antibiotic efficacy and vaccine strategy (1,2). Molecular and immunological tests have been used in the diagnosis of CE. IHA (Immune Hemagglutination), one of the immunological tests, is based on the antigen

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holding properties of tannic acid-sensitized erythrocytes, as a result of change in their surface tension. With a varying sensitivity of 80-94%, IHA has been considered as the most sensitive and easy to apply test in the diagnosis of cystic *Echinococcus* (7). Polymerase Chain Reaction (PCR) is the enzymatic reproduction of a DNA fragment located in between two oligonucleotide primers. One of the primers is complementary to the one chain of the DNA molecule in one side of the target sequence, and the second primer is complementary to the other chain of the DNA molecule on the other side of the chain sequence. Primers synthesize the sequence by the help of the DNA polymerase that is in between them. PCR based techniques are sensitive techniques used in the diagnosis of *Echinococcus* (8,9). Many new methods have been developed since the invention of the PCR technique [Multiplex PCR, nested PCR, arbitrary-primed PCR (AP-PCR), real-time PCR (qPCR), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)] (10). The most commonly used molecular techniques in the determination of genetic diversity of the *Echinococcus* species are PCR, RFLP, PCR-RFLP, RAPD-PCR (Random Amplified Polymorphic DNA), PCR-SSCP (PCR-Single Stranded Conformation Polymorphism) and DNA sequence analysis. DNA sequence analysis is considered as a gold standard technique among all the molecular techniques, although it requires specialty and interpretation of the results is difficult (2,6). The aim of this study was to evaluate the reproduction of *E. granulosus* DNA using the PCR technique and the genotypical characterization of it by DNA sequence analysis and to scrutinize the results in terms of clinical and pathological aspects.

MATERIAL and METHODS

This study was conducted prospectively at the Department of General Surgery of the Cerrahpaşa School of Medicine, University of İstanbul-Cerrahpaşa, between January 2015 and June 2016 in 30 patients who were operated on using different techniques

for CE diagnosed clinically, radiologically and serologically. The various surgical techniques used in this study were defined in previous studies, and surgical techniques that would not result in postoperative recurrence were preferred (11,12). Prior to the start of the study, ethics board approval was obtained from the Ethical Committee of Cerrahpaşa School of Medicine (approval number and date: 83045809- 604.01.02/07.12.2014) and informed consents from the patients were obtained. Patients with an age of less than 18 years were excluded from the study. Prior to the study, age, sex, additional disease or history of previous surgery, history of drug use, family history, occupation, place of birth, history of previous cystic *Echinococcus* disease or treatment received for it if applicable, history of CE in the relatives, location of the cyst, radiological findings and laboratory results of the patients were evaluated and recorded.

Sample Collection

Cyst samples (protoscolex, germinative membrane and laminar membrane) were stored at -80°C in 70% alcohol (Figure 1).

DNA Extraction

The cyst samples were washed several times in order to clear the alcohol. Cyst samples of 25-50 mg each were divided into multiple pieces, as small as possible. DNA extraction was performed using a commercial tissue extraction kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany).

Polymerase Chain Reaction

PCR method was applied as described previously by Bowles et al. (13) to the extracted tissue samples in order to analyze *E. granulosus* DNA by using primer sequences that belong to the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene region of the *E. granulosus* (Table 1). To this end, 1 mL forward primer (50 pmol/mL), 1 mL reverse primer (50 pmol/mL), 5 mL 10 x reaction buffer, 3 mL 25 mM MgCl₂, 1 mL dNTP, (200 mM deoxynucle-

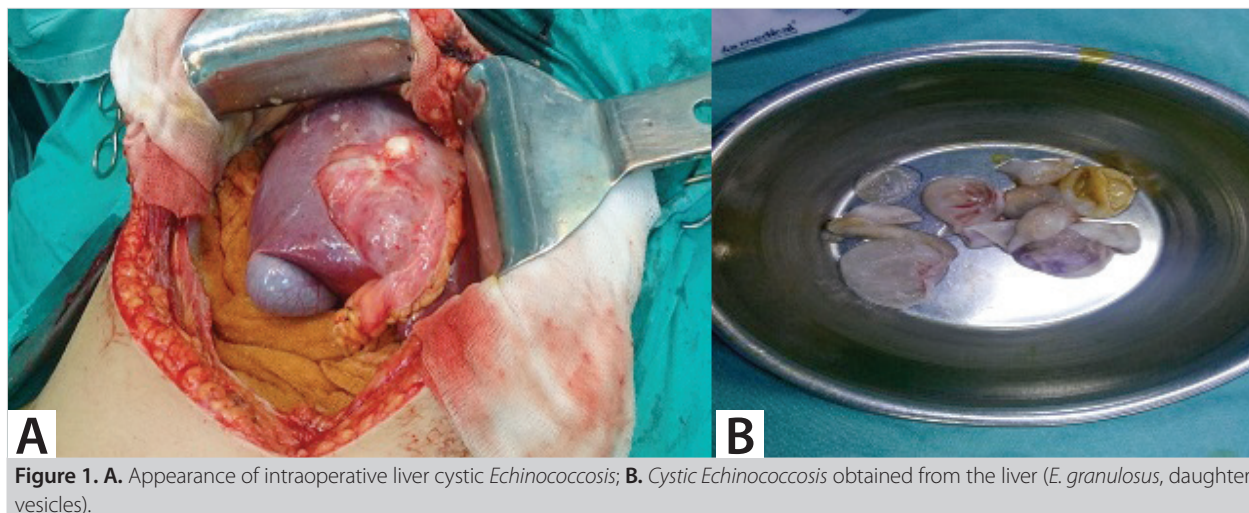


Figure 1. A. Appearance of intraoperative liver cystic *Echinococcus*; **B.** Cystic *Echinococcus* obtained from the liver (*E. granulosus*, daughter vesicles).

Table 1. Primer sets used in the study

Name of the Primer	Region	Sequence (5'-3')	Product Dimension
F-JB 3	Sense CO1	5'ttt ttt ggg cat cct gag gtt tat 3'	
R-JB 4,5	Anti-sense CO1	5'taa aga aag aac ata atg aaa atg 3'	446 bp.*
Beta-globin	Sense	5'aca caa ctg tgt tca cta gc 3'	
	Anti-sense	5'gga aaa tag acc aat agg ctg 3'	251 bp.*

*bp: Base pair.

otide triphosphate for each) (Fermentase®, Lithuania), 0.25 mL Taq DNA polymerase (Fermentase®, Lithuania) and a 28.75 mL mixture containing water and no DNase-RNasef was prepared. 10 mL of extraction product was added to the 40 mL mixture.

Amplification was performed as follows: Initially, the mixture was denaturated for two minutes at 95°C, and then 30 cycles were applied, with 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C. The final lengthening was performed at 72°C for eight minutes. The amplification products were viewed under UV light through application in a 10 mL/mL ethidium bromide added 1.5% agarose gel. All the samples were analyzed for the presence of inhibitors using beta-globulin primers.

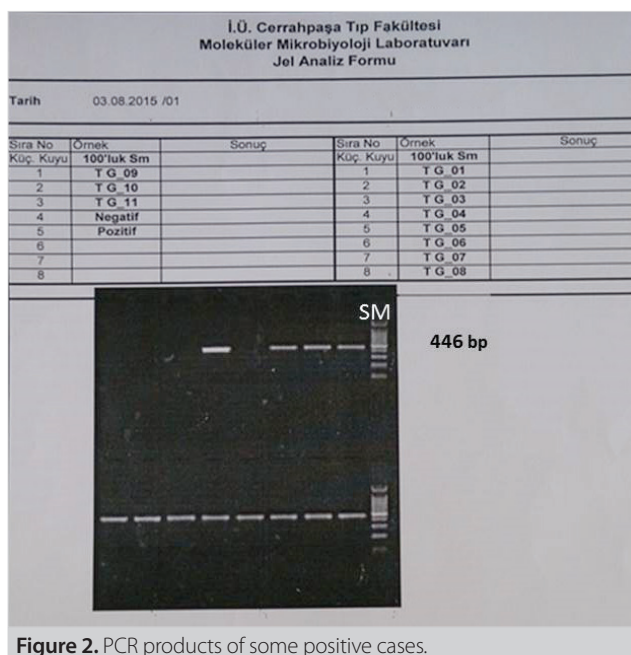
Genotype Determination

Genotype determination was performed in cases in which *E. granulosus* DNA positivity was detected. In order to determine the genotype, a commercial purification kit (High Pure PCR Product Purification kit, Roche Diagnostic, GmbH, Germany) was used, positive PCR products were purified and cycle-sequencing was performed using a big-dye terminator kit (ABI®, USA). Subsequently, the cycle sequencing products were cleared with the sephadex G-50 fine column method and analyzed in an automated DNA sequencing device (ABI®, 310). The obtained sequences were prepared using "Bioedit software (Hall, 1999)" and were compared with the sequences in GenBank™.

RESULTS

CE involved the liver in all 30 patients. Among the patients 11 were male (36.6%) and 19 were female (63.3%) with a mean age of 43.7 years. The location of the cyst was the right lobe of the liver in 24 patients (80%) and left lobe in six patients (20%). IHA tests performed in the preoperative period were positive in 22 (73.4%) and negative in eight (26.6%) patients. Among the eight IHA negative patients, the diagnosis of CE was proven radiologically and pathologically in seven (23.3%).

E. granulosus DNA was found to be positive in 29 out of the 30 patients in the study. The DNA sequence analysis method was used in the reproduced and purified isolates. From a comparison of the sequence analysis with the reference sequences (<http://www.ncbi.nlm.nih.gov/BLAST/BLASTdatabases.html>), all 29 isolates were determined to be the G1 (sheep) species.

**Figure 2.** PCR products of some positive cases.

An approximately 446 bp band was formed as a result of reproduction of *E. granulosus* using primer sequences of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene region using the PCR method. The band profiles of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene region of the positive controls and the samples following electrophoresis can be seen in Figure 2.

DISCUSSION

E. granulosus is a small parasite, 2-6 mm in length, that can be transmitted by its eggs and through the ingestion of contaminated food, but also through the skin. The hatching embryo (oncosphere) in the intestine attaches to the mucosa and produces cystic *Echinococcus* by passing to the liver through the gastrointestinal venous circulation and portal system. If the oncosphere is unable to attach here, it may pass to the lungs through the pulmonary artery, and CE may develop either in the lungs or in other organs (spleen, peritoneum, kidney, bone, orbital space, brain, heart and reproductive organs) (1,7).

E. granulosus is quite common worldwide, and is a very important cestode in terms of public health since it causes serious

zoonotic infestations. It produces structural and functional disorders in various tissues and organs of animals, leading subsequently to economic losses (14). Different species are important in the lifecycle of the parasite and also in host selectivity, rate of development, pathogenicity, antigenicity, sensitivity to chemotherapeutics and transmission properties, as well as the epidemiology and controllability of the disease. The determination of the genotype distribution in endemic areas of the disease, and successful application of control and eradication programs in such areas is, therefore, extremely important (2,15).

The most common genotype in humans and animals in this country and around the world is considered to be the G1 (sheep) species, based on genoepidemiological studies that have made use of molecular techniques (16,17). The G1 genotype has been reported to be the most frequently seen genotype in humans and animals in Mediterranean countries on the same continent as Turkey (18,19). Elsewhere, the G6 camel species in Egypt and the G7 pig species in Poland have been reported to be more common (4,20).

Geographical circumstances and social structure affect the prevalence of the *Echinococcus* type in this region. In Turkey, *Echinococcus* infestations are most often seen in sheep and dogs, and these animals and the G1 species are also the most common and most frequent origin of human infections (21).

Studies of hydatid cysts in humans and animal origin studies are quite scarce in Turkey. Vural et al. (22) identified G1 (sheep) and G3 (bovine) genotypes following a sequence analysis of the CO1 gene region of *E. granulosus* isolates obtained from sheep and bovines in different cities in all regions of the country. Simsek and Eroksuz (23) have reproduced the agent using the mitochondrial CO1 gene region in a cyst sample obtained from wild Anatolian sheep (*Ovis gmelinii* Anatolica) and determined it to be the G1 species based on a sequence analysis. Utuk et al. (24) on the other hand, found that all of the *E. granulosus* isolates were of the G1 genotype in their wide series involving humans, dogs, camels, sheep and bovine. Engin et al. (25) determined G1 species in all of the human isolates in their study of patients from different regions of Turkey using primers belonging to the mitochondrial CO1 gene region in the cyst samples. Snabel et al. (26), on the other hand, identified for the first time the presence of the G7 (pig) species in Turkey in sheep and human isolates collected from various cities in the Aegean region. Eryildiz et al. (27) carried out a sequence analysis of the mitochondrial cytochrome oxidase c subunit one and NADH dehydrogenase subunit one gene regions in their molecular study, and identified the presence of two different genotypes in their study, being G1 and G7.

In the present study, the presence of the G1 (sheep) species was detected in all 29 of the samples in which the sequence analysis of the mitochondrial CO1 gene region of the *E. granulosus* could be performed among the 30 samples of CE located in the liver. This finding concurs with those of studies in which the G1 (sheep) species was identified as the most common genotype in this country and around the world.

CE is still a threat to public health in Turkey, as an endemic region. Screening immigrants through field studies with rapid tests is required in regions where immigration from neighboring countries where the disease is endemic, and where the prevalence of the disease is high. Further molecular tests should be conducted to determine the species when necessary, for which the subject should be evaluated by public health specialists and molecular epidemiologists in collaboration, and risk maps of Turkey's regions should be prepared based on risk analysis methods. The extent and nature of the precautions taken to control the disease may vary based on the characteristics of the species. Large-scale genoepidemiological studies should be carried out and control and eradication programs should be launched, either periodically or during extraordinary mass immigration events, to bring cystic *Echinococcosis* under control.

CONCLUSION

CE is a significant public health problem in several countries around the world. Many *E. granulosus* subtypes have been identified through molecular analyses, and the most common species was found to be the G1 sheep species in the present study performed in the Cerrahpaşa Medical Faculty. Studies should be conducted in this region into the sheep-dog cycle, as the main path of transmission, as a means of preventing human transmission in rural areas and on animal farms in the region. Studies involving the PCR technique in different regions would aid in the prevention of transmission and distribution of CE by determining the predominant subtypes in a particular region. A vaccine developed for the most commonly seen genotypes identified through larger scale geno-epidemiological studies may serve as a solution for this global public health problem.

Ethics Committee Approval: This study was approved by Cerrahpaşa Faculty of Medicine Research Ethics Committee (Decision no: 442684, Date: 07.12.2016).

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ORİJİNAL ÇALIŞMA-ÖZET

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Karaciğer hidatik kisti hastalarında polimeraz zincir reaksiyonu (PZR) yöntemi ile ekinokok DNA'sının araştırılması

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ÖZET

Giriş ve Amaç: Bu çalışmada insandan izole edilen karaciğer hidatik kistlerinde *Echinococcus granulosus* DNA ve genotiplerinin belirlenmesi amaçlandı.

Gereç ve Yöntem: Çalışma prospektif randomize olarak, 2015 Ocak-2016 Haziran tarihleri arasında İstanbul Üniversitesi, Cerrahpaşa Tıp Fakültesi Genel Cerrahi Anabilim Dalı'nda karaciğer hidatik kisti tanısı ile ameliyat edilen 30 hasta üzerinde gerçekleştirildi. Ameliyat sırasında elde edilen kist örneklerinde (protoskoleks ve/veya germinatif membran) Polimeraz Zincir Reaksiyonu (PZR) yöntemiyle *E. granulosus* DNA'sı araştırıldı ve pozitif bulunan örneklerde dizi analizi yöntemiyle genotip tayini yapıldı. Aynı zamanda ameliyat öncesinde hastaların kanlarında indirekt hemaglutinasyon testi (IHA) ile antikor varlığı araştırıldı.

Bulgular: İncelenen 30 karaciğer hidatik kisti örneğinin 29'unda *E. granulosus* DNA'sı saptandı. 29 hidatik kist örneğinin tamamının G1 (koyun) suşu olduğu görüldü. Ayrıca 22 hastada IHA pozitif, sekiz hastada ise negatif idi.

Sonuç: Çalışmamızda en sık görülen hidatik kist genotipi G1 suşudur. Bölgesel olarak yaygın görülen genotiplere uygun geliştirilebilecek bir aşının bu morbidite ve mortalite kaynağı hastalığın önlenmesinde etkili olacağını düşünüyoruz.

Anahtar Kelimeler: Karaciğer hidatik kisti, *Echinococcus granulosus*, genotip, DNA, PZR, Western-Blot

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