Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

Genotyping of *Echinococcus granulosus* Human Isolates Obtained from the Puncture, Aspiration, Injection, and Re-Aspiration (PAIR) Process in Türkiye

Önder Akkaş [1], Mesut Özgökçe [2], *Selahattin Aydemir [3], İlyas Dündar [2], Abdurrahman Ekici [3]

1. *Department of Microbiology, Medical Faculty, Erzincan Binali Yıldırım University, Erzincan, Türkiye*
2. *Department of Radiology, Medical Faculty, Van Yüzüncü Yıl University, Van, Türkiye*
3. *Department of Parasitology, Medical Faculty, Van Yüzüncü Yıl University, Van, Türkiye*

Received 04 Jul 2023
Accepted 14 Oct 2023

Keywords:
Echinococcus granulosus;
Genotype;
Human;
Türkiye

***Correspondence Email:**
saydmr23@gmail.com

Abstract

Background: *Echinococcus granulosus* is spread by the excretion of cystic organs into the environment. The dog is infected via eating the cystic organ. It then contaminates the environment with eggs of *E. granulosus*, which are infective to humans and animals. We aimed to determine the *E. granulosus* genotypes that cause infection in humans in the Van region, Türkiye.

Methods: Sixty patients between 18 and 100 years of age, who underwent the puncture, aspiration, injection, re-aspiration (PAIR) procedure in the Department of Radiodiognastics of Van Yüzüncü Yıl University, Van, Türkiye were included in the study. PAIR fluids were examined microscopically and DNA was isolated from the fluids. After DNA isolation, polymerase chain reaction (PCR) was performed using primers that amplify the *E. granulosus NADH dehydrogenase subunit 1* (*NAD1*) gene region. After sequence analysis of the PCR amplicons, Basic Local Alignment Search Tool (BLAST) was performed.

Results: In the microscopic examination, protoscolex or hook was detected in 42 (70%) of the samples. DNA was successfully extracted from all of the cyst fluids containing protoscolex and hook, and the *NAD1* gene region was PCR-amplified. After using BLAST, all of the samples were determined to be an *E. granulosus* sensu stricto G1 genotype. Sequence comparison revealed that four (9.5%) isolate sequences showed single nucleotide polymorphism (SNP). Sequences of isolates with SNP submitted to the GenBank with accession numbers OR565864 to OR565867.

Conclusion: *E. granulosus* s.s. G1 genotype, known as sheep strain, is common in human hydatid disease in the Van region of Türkiye.

Introduction

Echinococcus granulosus, a cestode belonging to the Taeniidae family, is the causative agent of cystic echinococcosis (CE), a common zoonotic disease worldwide. Canines are the final host for these tapeworms and herbivores/omnivores are intermediate hosts. Human infection occurs incidentally by ingestion of *E. granulosus* eggs. In humans, it causes the development of fluid-filled hydatid cysts localized in the liver and lungs, and to a lesser extent in the abdominal cavity, muscle, heart, bone, and nervous system (1,2).

The *Echinococcus* genus includes five species including *E. granulosus* sensu lato, *E. multilocularis*, *E. shiquicus*, *E. vogeli*, and *E. oligarthra*. *E. granulosus* s.l. is a complex of genotypes that cause CE in humans and animals. Five species complexes of *E. granulosus* s.l. are now known Because of phylogenetic analysis: *E. granulosus* sensu stricto (G1 and G3 genotypes), *E. equinus* (G4 genotype), *E. ortleppi* (G5 genotype), *E. canadensis* (G6/G7, G8/G10 genotypes), and *E. felidis*. The highest number of human cases worldwide were found as the sheep strain, that is, the G1 genotype. Genotype G2 is no longer recognized as a valid genotype but as a microvariant of G3 (3,4).

This wide diversity of genotypes also influences several characteristics of the parasite, such as drug susceptibility, including its life cycle, transmission, pathogenicity, and biochemical characteristics. It has been noted that each genotype of *E. granulosus* tends to infect a certain intermediate host and certain organs of the body. Therefore, in a given geographic area, the dominant genotypes of *E. granulosus* can be determined, and appropriate planning can be made to prevent transmission of parasites and transmission to humans between intermediate and definitive hosts (3).

Chemotherapy-related open surgery was the treatment of choice for the treatment of E. granulosus, but a mini-invasive procedure called puncture, aspiration, injection, reaspiration (PAIR) was recommended by a Tunisian team in 1986. Today, it is an accepted method for the treatment of hydatid cysts by the WHO (5).

This study was carried out to determine the *E. granulosus* genotypes that cause infection in humans in the Van region of Türkiye.

Materials and Methods

Ethical approval

Ethical approval for this study was obtained from the Clinical Research Ethics Committee of Yüzüncü Yıl University, Faculty of Medicine, with the decision no. 04 dated 15/12/2021.

Samples collection

Sixty patients, between 18 and 100 years of age, who underwent the PAIR procedure in the Department of Radiodiognastics of Yüzüncü Yıl University, Faculty of Medicine, were included in the study. The fluids obtained from the patients because of the PAIR procedure were centrifuged at 4000 rpm for 5 min and the underlying precipitate was used.

Microscopic examination

In order to distinguish between sterile cysts and fertile cysts, the precipitate obtained because of centrifugation was examined under a microscope. The 10-lens objectives of the light microscope were used to detect protoscolices. The sediments in which no protoscolex could be detected were examined with 40 and 100 lenses to investigate the presence of hooks. After the examination, the samples were stored at –20 °C until DNA isolation.

DNA isolation

After the materials stored at –20 °C were thawed at room temperature, total DNA isolation was performed with the Genomic DNA Purification Kit (Thermo Scientific GeneJET

K0722, Lithuania) in accordance with the kit manufacturer's instructions.

Conventional PCR

In the polymerase chain reaction (PCR) method, primers NAD1F 5' - TATTCT-CARTYTCGYAAGGGHCC - 3' and NAD1R 5' - AACCATTTCTTGAAGTTAACAGCAG-CATC - 3' amplifying the *E. granulosus NADH dehydrogenase subunit 1* (*NAD1*) gene region were used (6). The reaction was adjusted to a total volume of 50 µL, containing 25 µL of Taq 2x Master Mix (with 12.5 mM $MgCl_2$) (Ampliqon), 0.5 mM of MgCl2, 0.2 µM from each primer (Sentegen), and 4 µL of sample DNA.

Reactions were run on an Applied Biosystems SimpliAmp Thermal Cycler PCR instrument. PCR was programmed for 45 cycles of 40 s at 95 °C, 40 s at 50°C, and 65 s at 72 °C. In addition to the PCR process, a 4-min denaturation step at 95 °C was performed before the first cycle, as well as an extension phase at 72 °C for 10 min following the last cycle. In order to display the results, 15 µL of the obtained reaction products were run in gel electrophoresis and visualized in the UVP Gel documentation system.

DNA Sequence Analysis of the partial NAD1 Gene Region

The PCR product of the mitochondrial *NAD1* gene region was sent to BM Labosis (Ankara/Türkiye) for bidirectional DNA sequence analysis. Blast (www.ncbi.nlm.nih.gov/BLAST/) was performed using reference genotypes (Table 1) from GenBank. Reference gene sequences and bidirectional sequence analysis results were compared using the SnapGen program. Base changes in both strands of the same sample were considered single nucleotide polymorphism (SNP). Changes seen in a single chain were evaluated as a read error in sequence processing and were corrected according to the reference sequence. Phylogenetic analysis was performed using nad1 sequence data representing nucleotide variations seen in the present study, sequences for genotypes of *E. granulosus* sensu lato from previous studies as well as *Taenia saginata* as the outgroup. The phylogenetic analysis was constructed according to the neighbor-joining (NJ) method using MEGA11 software. The distances were computed using the Maximum Composite Likelihood method.

Table 1: Reference sequences of the Echinococcus granulosus genotypes used in the study.

Genotype	*Genbank accession number*	*Reference*
G1	KU925392	Kinkar et al. (7)
G3	KJ559023	Wang et al. (8)
G4	KT363809	Chaabane-Banaoues et al. (9)
G5	KT363810	Chaabane-Banaoues et al. (9)
G6	KT363811	Chaabane-Banaoues et al. (9)
G7	KT363812	Chaabane-Banaoues et al. (9)
G8	AB235848	Nakao et al. (10)
G10	AB745463	Nakao et al. (11)

Results

Overall, 39 (65%) of the sixty patients who received PAIR fluid were female and 21 (35%) were male. In the microscopic examination, protoscolex (Fig. 1) or hook (Fig. 2) was detected in 42 of the samples (70%). DNA was successfully extracted from all of the cyst fluids containing protoscolex and hook, and the *NAD1* gene region was amplified by PCR. When the sequence results were examined, it was determined that the gene region with a length of 777 bp was free of errors for all of the samples. After performing BLAST, all of the samples were determined to be a *E. granulosus* s.s. G1 genotype.

Sequence comparison revealed that 38 (90.5%) isolates showed 100% homology with the G1 (KU925392.1) genotype, while the other four (9.5%) isolate sequences showed SNP (Figs. 3 and 4). Sequences of VANG1.1, VANG1.2, VANG1.2, VANG1.2 isolates with SNP were submitted to the GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) with accession numbers of OR565864, OR565865, OR565866 and OR565867, respectively

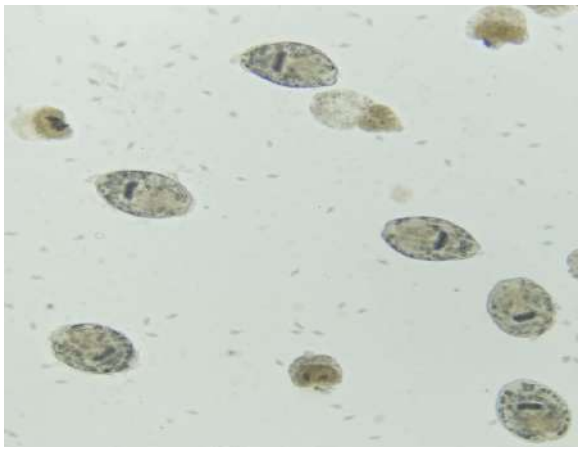

Fig. 1: Protoscoleces image of a human liver *E. granulosus* isolate of this study (X400)

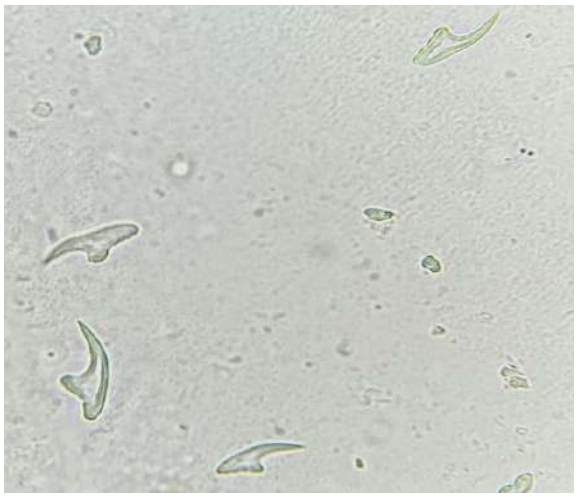

Fig. 2: Hooks image of a human liver *E. granulosus* isolate of this study (X1000)

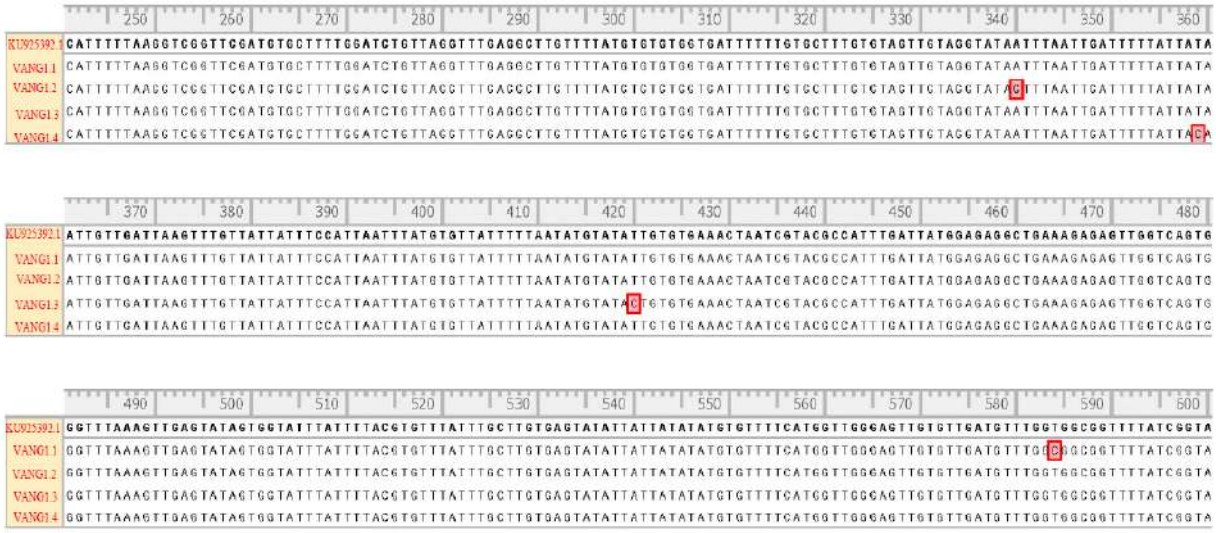

Fig. 3: Sequence comparison of four isolates of *E. granulosus* G1 genotype with SNP from this study with G1 reference sequence (KU925392).

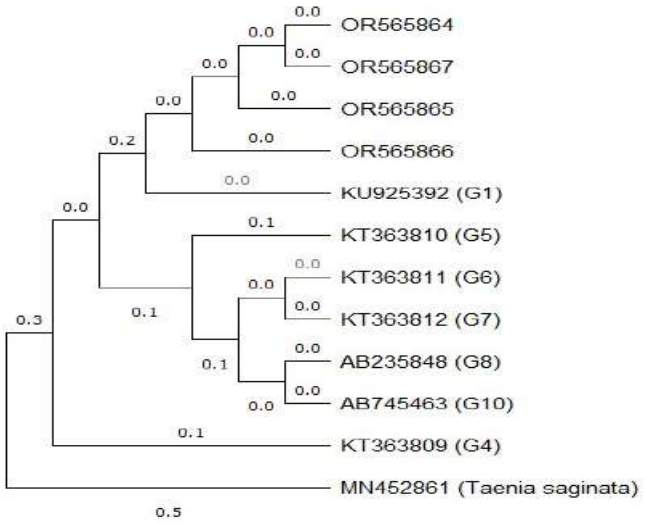

Fig. 4: Genetic relationships of four isolates of *E. granulosus* from this study and reference sequences for *E. granulosus* sensu lato selected from previous studies as well as *T. saginata* as the outgroup using the Neighbor-Joining method in MEGA11 software

Discussion

Hydatid cyst disease is an infection in which humans are an incidental intermediate host and is caused by the larvae stage of *E. granulosus*. The disease occurs most commonly in the liver, and secondarily in the lungs. There are various treatment methods for this disease. Although surgery is the most common and well-known treatment method, it is associated with significant morbidity and long hospital stay. With the developing technology, optimal

treatment methods based on minimally invasive procedures such as ultrasound-guided PAIR have been developed (12). PAIR treatment together with oral anthelmintic therapy is very effective and gives better results than surgery in Gharbi type I-III cysts (13). Cyst hydatid materials (isolates) can be obtained during surgical removal of the cyst as well as during the PAIR procedure (14). During the PAIR procedure, if the cyst is fertile, hydatid sand containing protoscolex is obtained together with the cyst fluid. Molecular studies can be done by isolating DNA from the protoscolex obtained. Most studies of the genotyping of CE cases have used cyst materials obtained by surgical procedure (15-18). In one study, cyst materials obtained by surgical procedure and PAIR procedure were used together (14). With this study, the cyst isolates obtained using the PAIR procedure could also be genotyped. However, it is a disadvantage that DNA cannot be obtained from sterile cyst fluids obtained using the PAIR procedure. In this study, because the cyst fluid of 18 (30%) patients was sterile, DNA could not be obtained from these samples.

The genotyping of human CE cases plays an important role in the formulation of control strategies to prevent transmission of this parasite. Genotype variation in parasites varies in host specificity and transmission dynamics (19). Worldwide, the *E granulosus* s.s. genotypes most commonly associated with CE in humans are the G1 and G3 genotypes (20).The G1 genotype is known as sheep strain. Although the G1 genotype can infect other intermediate hosts, such as cattle, goats, and dogs, the fertility rate of cysts in these animals is either low or the cysts are not fertile at all (21). In Türkiye, different genotypes have been detected, but the G1 genotype was determined as the dominant strain. In Istanbul, all of human CE isolates were the G1 genotype (21). In Ankara, 94.5% of the human CE isolates belonged to the *E. granulosus* s.s (G1 and G3) complex, and 5.5% to the *E. canadensis* (G6/7) complex (22). In Aydın, 75% of their human CE isolates were G1 and 25% were *E. canadensis* (G6/G7) genotype (15). In another study, 95% of the human CE isolates collected in Van Province were G1 and 5% (one) were the G3 genotype (3). In Konya, a human isolate was the G4 genotype in addition to G1 and G3 (23). In this study, all of the isolates were determined to be the G1 genotype and point mutations were detected in the NAD1 gene region in four isolates.

In Van, 70% of the total surface area is meadow-pasture and the land conditions are particularly suitable for sheep-goat breeding. For this reason, sheep breeding is widely practised under extensive conditions (24). In addition to intensive sheep breeding, the prevalence of CE in sheep raised in Van was too high to be underestimated. In one study, CE was detected in 46.4% of 525 sheep slaughtered in Van slaughterhouses (25). On the other hand, in Türkiye, mostly sheep are slaughtered during the feast of sacrifice. The disease is still common in Türkiye due to reasons such as not properly destroying the cystic organs in the slaughter and throwing them out for the dogs to eat (15).

In light of this information and the determination of the G1 genotype, which is known only as a sheep strain in this study, sheep breeding has an important place in hydatid cyst disease seen in people living in the Van region.

Conclusion

The *E. granulosus* s.s. G1 genotype, known as sheep strain, is common in the Van region, that sheep breeders should be made aware of hydatid cyst eradication, slaughtering should be done in a controlled manner, and suspicious organs should be destroyed appropriately.

Funding

None.

Conflict of interest

The authors declare no conflict of interest.

References

1. Khademvatan S, Majidiani H, Foroutan M, Tappeh KH, Aryamand S, Khalkhali H. *Echinococcus granulosus* genotypes in Iran: a systematic review. J Helminthol. 2019;93(2):131-8.
2. Akkaş O, Uslu H, Yılmaz I, Aydın Y, Korkut E, Yılmaz A. Serological screening of patients diagnosed with alveolar *Echinococcus* disease in their home regions. Iran J Parasitol. 2022; 17(4):554-561.
3. Barazesh A, Sarkari B, Shahabi S, et al. Genetic diversity of *Echinococcus granulosus* isolated from humans: A comparative study in two cystic echinococcosis endemic areas, Turkey and Iran. Biomed Res Int. 2020;2020.Artn 305419510.1155/2020/3054195.
4. Casulli A, Massolo A, Saarma U, Umhang G, Santolamazza F, Santoro A. Species and genotypes belonging to *Echinococcus granulosus* sensu lato complex causing human cystic echinococcosis in Europe (2000-2021): a systematic review. Parasit Vectors. 2022;15(1):109.10. DOI:1186/s13071-022-05197-8.
5. Bellasri S. Double puncture-aspiration-injection-reaspiration (dPAIR) in hydatid cysts management: Report of 10 cases. Sch J Med Case Rep. 2021;3:227-30.
6. Cláudio Roque. Estudo molecular de amostras de *Echinococcus granulosus* mm Portugal [Thesis]. DOI: 10.13140/2.1.4430.0169.
7. Kinkar L, Laurimae T, Simsek S, et al. High-resolution phylogeography of zoonotic tapeworm *Echinococcus granulosus* sensu stricto genotype G1 with an emphasis on its distribution in Turkey, Italy and Spain. Parasitology. 2016;143(13):1790-801.
8. Wang N, Xie Y, Liu T, et al. The complete mitochondrial genome of G3 genotype of *Echinococcus granulosus* (Cestoda: Taeniidae). Mitochondrial DNA Part A. 2016;27(3):1701-2.
9. Chaabane-Banaoues R, Oudni-M'rad M, M'Rad S, Amani H, Mezhoud H, Babba H. A novel PCR-RFLP assay for molecular characterization of *Echinococcus granulosus* sensu lato and closely related species in developing countries. Parasitol Res. 2016;115(10):3817-24.
10. Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology. 2007;134(Pt 5):713-22.
11. Nakao M, Yanagida T, Konyaev S, et al. Mitochondrial phylogeny of the genus *Echinococcus* (Cestoda: Taeniidae) with emphasis on relationships among *Echinococcus canadensis* genotypes. Parasitology. 2013;140(13):1625-36.
12. Kaniyev S, Baimakhanov Z, Doskhanov M, Kausova G, Baimakhanov B. Recent treatment results of liver echinococcosis by pair method (puncture, aspiration, injection, reaspiration). Georgian Med News. 2020(308):11-4.
13. Rajesh R, Dalip DS, Anupam J, Jaisiram A. Effectiveness of puncture-aspiration-injection-reaspiration in the treatment of hepatic hydatid cysts. Iran J Radiol. 2013;10(2):68-73.
14. Aaty HA, Abdel-Hameed D, Alam-Eldin Y, et al. Molecular genotyping of *Echinococcus granulosus* in animal and human isolates from Egypt. Acta Trop. 2012;121(2):125-8.
15. Babaoğlu AO, Soyder A, Malatyalı E, Ertuğ S, Ertabaklar H. Genotyping of *Echinococcus granulosus* isolates by sequencing of *mitochondrial cytochrome C oxidase* subunit 1 *(cox1)* gene in Aydin. Mikrobiyol Bul. 2018;52(2):198-205.
16. Eryildiz C, Sakru N. Molecular Characterization of human and animal isolates of *Echinococcus granulosus* in the Thrace Region, Turkey. Balkan Med J. 2012;29(3):261-7.10.5152/balkanmedj.2012.072.
17. Hammad SJ, Cavallero S, Milardi GL, Gabrielli S, Al-Nasiri FS. Molecular genotyping of *Echinococcus granulosus* in the North of Iraq. Vet Parasitol. 2018;249:82-7.
18. Nikmanesh B, Mirhendi H, Ghalavand Z, et al. Genotyping of *Echinococcus granulosus*

isolates from human clinical samples based on sequencing of mitochondrial genes in Iran, Tehran. Iran J Parasitol. 2014;9(1):20-27.

19. Sharma M, Sehgal R, Fomda BA, Malhotra A, Malla N. Molecular characterization of *Echinococcus granulosus* cysts in north Indian patients: identification of G1, G3, G5 and G6 genotypes. PLOS Negl Trop Dis. 2013;7(6):e2262.
20. Kinkar L, Laurimäe T, Acosta-Jamett G, et al. Distinguishing *Echinococcus granulosus* sensu stricto genotypes G1 and G3 with confidence: a practical guide. Infect Genet Evol. 2018;64:178-84
21. Sevgi E, Suat S, Pelin Y, et al. Genotypic characterisation of *Echinococcus granulosus* isolated from human in Turkey. Afr J Microbiol Res. 2010;4(7):551-5.
22. Orsten S, Ciftci T, Azizova A, et al. Investigation of the relationship between CE cyst characteristics and genetic diversity of *Echinococcus granulosus* sensu lato in humans from Turkey. Parasitology. 2020;147(14):1712-7. 10.1017/S0031182020001535.
23. Macin S, Orsten S, Samadzade R, Colak B, Cebeci H, Fındık D. Human and animal cystic echinococcosis in Konya, Turkey: Molecular identification and the first report of *E. equinus* from human host in Turkey. Parasitol Res. 2021;120(2):563-8.
24. Yıldız A, Aygun T. Activities of small ruminant husbandry and general problems in Van central district: I. structural features of enterprises. JASP. 2021; 4(1):23-36.
25. Oğuz B, Değer S. Cystic echinococcosis and cysticerci of *Taenia hydatigena* in cattle and sheep slaughtered in a Van local slaughterhouse. Turkiye Parazitol Derg. 2013;37:186-9.