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Evaluation of the Molecular Properties of *Echinococcus granulosus* Isolates from Various Hosts in Şanlıurfa Province[*]

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Abstract: Cystic *echinococcosis* (CE), which is raised by the larvae of *Echinococcus granulosus* (*E. granulosus*), is single of the world's most significant zoonoses, affecting both humans and animals. In this labor, it was the goal to perform genotyping of hydatid cyst stocks collected from various regions of Şanlıurfa and diverse middle hosts by molecular methods. A total of 197 hydatid cyst samples were obtained, including 40 pre-diagnosed human samples with paraffin, Türkiye 140 sheep, and 17 cattle samples. After microscopic examination, 33 human isolates and 80 sheep cyst are regarded fertile because they contain more than 250 protoscolex and/or scolex hooks. PCR-RFLP technique was used to determine the genetics of fertile isolates by analyzing the ribosomal DNA internal transcribed spacer gene 1 (rDNA-ITS1) region of the samples. As a result of having the same band profile, it was found that all of the isolates were common sheep strain (G1). *Echinococcus granulosus* G1 strain was the reference material. This study demonstrated that the local sheep lineage of *E. granulosus* is the dominant genotype in Şanlıurfa province. It is thought to lead to further work on the epidemiology and ecology of parasites in animals in this region and neighboring countries.

Keywords: Cystic echinocystic, *Echinococcus granulosus*, genotype, PCR, RFLP.

Şanlıurfa İlindeki Çeşitli Konakçılardan *Echinococcus granulosus* İzolatlarının Moleküler Özelliklerinin Değerlendirilmesi[*]

Öz: *Echinococcus granulosus* larvalarının neden olduğu kistik ekinokokozis (KE), hem insanları hem de hayvanları etkileyen dünyadaki en önemli zoonozlardan birisidir. Suşlar arasındaki ara konak farklılıkları ve gelişimsel farklılıklar parazitle mücadeleyi ve kontrol çalışmalarını olumsuz yönde etkileyebilmektedir. Bu çalışmada Şanlıurfa'nın farklı bölgelerinden ve farklı ara konaklardan elde edilen kist hidatik materyallerinden moleküler yöntemlerle genotiplendirme yapılması amaçlanmıştır. Türkiye'nin Şanlıurfa ilindeki hastanemizde tanısı önceden konulmuş 40 tane parafinli insan numunesi ve mezbahanemizden alınan 140 koyun ve 17 sığır numunesi olmak üzere 197 hidatik kist örnekleri toplanmıştır. Bu izolatlarda fertil olan 33 parafinli insan izolatı ve 80 koyundan alınan kist sıvısında mikroskobik inceleme sonrası 250 üzeri protoskoleks ve/veya skoleks çengeli olduğundan fertil kabul edildi. *Echinococcus granulosus* G1 suşu referans metaryali olarak kullanıldı. Bu izolatların genetiğini belirlemek amacıyla örneklerin ribozomal DNA internal transcribed spacer gene 1 (rDNA-ITS1) bölgesi PCR-RFLP tekniğiyle incelenmiştir. Aynı band profiline sahip olması sonucu izolatların hepsinin yaygın koyun suşu (G1) olduğu belirlenmiştir. Bu çalışma ile Şanlıurfa'da *E. granulosus* 'un evcil koyun suşunun baskın genotip olduğu gösterilmiştir. Elde edilen sonuçların bu bölge ve komşu ülkelerde hayvanlardaki parazitlerin epidemiyolojisi ve ekolojisi ile ilgili ileri çalışmalara yol göstereceği düşünülmektedir.

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Anahtar kelimeler: *Echinococcus granosus*, genotip, Kistik ekinokistik, PCR-RFLP.

INTRODUCTION

Cystic echinococcosis [CE], also given as hydatid cyst, is a zoonotic disease caused by the larvae of the *Echinococcus granulosus*. Cystic echinococcosis is common worldwide and in Türkiye, particularly in regions where animal husbandry is prevalent, and it causes both economic and health problems for people. The disease is also called cystic echinococcosis, hydatid echinococcosis, and hydatidosis. According to current data, the *E. granulosus sens lato* have been demarcated 5 species in 10 genotypes; *E. granulosus sensu stricto* (domestic sheep strain [G1], Tasmanian sheep strain [G2], buffalo strain [G3]), *E. equinus* (horse strain [G4]), *E. ortleppi* (cattle strain [G5]), *E. canadensis* (camel strain [G6], pig strain [G7], variant of pig strain [G9] and cervid strain [G8 and G10]), and *E. felidis* (lion strain) (Wen et al., 2019).

The life cycle of *E. granulosus sensu stricto* is dependent on carnivore predator as definitive host and prey mammals as intermediate host association. Humans are accidental hosts. They infect with parasite eggs are excreted with feces of definitive hosts, especially dogs. The larval forms, known as metacestodes, settle in different organs, especially the liver and lungs. Cyctic echinococcosis is highly endemic in many regions of the world; western China, Central Asia, Medditerian countries, Eastern Africa, and South America (Rogan, 2001; Thompson & McManus, 2002; Unat et al., 1995).

Türkiye is highly endemic for CE. G1 and G3 are dominant strains, while G4, G6, and G7 are also reported in Türkiye (Borhani et al., 2021). This study aims to make genotyping of CE in humans and slaughtered animals in the Şanlıurfa region by PRC-RFLP method and to contribute to epidemiological studies.

MATERIAL AND METHOD

In this study, paraffinic samples [general surgery, urology, gastrology, etc. obtained from operating rooms] diagnosed with hydatid cysts in Şanlıurfa Mehmet Akif İnan Training and Research Hospital and Harran University Research and Application Hospital in 2015 and all parenchymatous organs, especially liver and lung, of sheep and cattle weekly obtained from Demir Meat Integrated Facilities Industry and Trade Joint Stock Company [DEM-ET] were examined.

During weekly trips to the slaughterhouse, at most two cyst samples were gathered from diseased sheep and cattle to determine the genotype of the various slaughtered herds. Organs with cysts were taken to the Microbiology Department Laboratory of the Faculty of Medicine at Harran University. In a quod way, a total of 197 hydatid cyst isolates were obtained, including those from 40 humans [age range: 40-70 years old, gender: 28 males and 12 females], 140 sheep, and 17 cattle.

Paraffinic samples of human fertile isolates (33) were examined. Isolates of sheep and cattle also were investigated.

Sterile samples were not included in the work, and 113 fertile samples were stored at -20°C in 70% ethyl alcohol until they were examined. Samples containing 250 or more protoscolex were extracted by performing extraction standardization.

The presence of protoscolex was evaluated using light microscopy on cyst fluid extracted with a sterile injector and tissue samples collected from the germinal membrane. Each cyst was examined as an individual isolate, and protoscolex and germinal membrane tissue samples were preserved at -20 ºC in 70% ethanol.

Since the success rate of deparaffinization of paraffin samples is very low, researchers who plan to conduct molecular studies on echinococcosis in the future should preferably use fresh samples and, if this is not possible, keep them in formaldehyde for a long time. It is recommended to protect their scolex along with their germinal centers without waiting.

Then, using the Genomic DNA Isolation Kit per the manufacturer's instructions, DNA was extracted from paraffin-embedded tissue and RTA tissue. The amount of DNA in the extracted products was measured by using a spectrophotometer. Primers BD1[5'- GTC-GTA-ACA-AGG-TTT-CCG-TA-3'] and 4S[5´ TCT AGA TGC GTT CGA [A/T] GTC GAT G 3´] were used to amplify the rDNA region that includes the flanking regions of BD1 and 4S. The PCR mixture consisted of 5 µl 10x PCR buffer, 4 µl of dNTP [2.5 mM], 50 mole each primer, 0.5 µl Taq DNA polymerase [5 U/ml], 10 µl template DNA, total volume completed 50 µl with sterile distilled water. The thermal cycler was programmed to amplify the ribosomal DNA-ITS1 gene region as follows: 35 cycles, 5 minutes at 94 °C for pre-denaturation, 1 minute at 95 °C for denaturation, 1 minute at 53 °C for binding, 1 minute at 72 °C for synthesis, and 5 minutes at 72 °C for final elongation.

Electrophoresis and evaluation: 8 µl of DNA size marker was loaded into the first well of a 1% agarose gel, while 15 µl of PCR product was loaded into the other wells and loaded into the gel. 0.5 x TBE was used as the electrophoresis buffer solution, and the experiment was conducted at 90 volts for 2 hours using an electrophoresis device. In the continuation of the study, samples with DNA size marker and band at the appropriate place according to reference G1 under UV light were used. PCR products determined to be positive for the ITS-1 gene were cut using

the AluI, RsaI, and TaqI enzymes. The content of the reaction was as follows: (PCR product: 15 µl, 10x restriction buffer: 5 µl, BSA: 0.5 µl, restriction enzyme: 0.4 µl and made up to 50 µl with sterile distilled water). Separate cuts were made with restriction enzymes. AluI and RsaI enzymes were hatched at 37 °C for 1-30 hours, whereas the TaqI enzyme was hatched at 65 °C for 1-30 hours. Cutting has been done. Electrophoresis and evaluation: At the end of the incubation, the products were loaded on 2% agarose gel. In the agarose gel, 8 ml of DNA size marker was loaded into the first well and 15 µl of cut products were loaded into the other wells. Two hours of electrophoresis was performed at 90 volts. After this period, the agarose gel was visualized under UV light. Additionally, Local Ethics Committee approval was received for the study.

RESULTS AND DISCUSSION

After microscopic examination, 113 [57.4%] out of 197 cyst samples were found fertile, including 33 human and 80 sheep samples (Table 1)

Table 1. Distribution of Cyst Samples from Humans and Animals by Fertility Status

	CYST NUMBER		
SPECIES	Fertile (%)	Sterile (%)	Total
Human	33(82.5)	7 (17.5)	40
Cattle	-	17(100)	17
Sheep	80 (57.14)	60(42.85)	140
Total	113(57.36)	84(42.63)	197

In fertile samples, a total of 60 isolates remained after removing hydatid cyst materials that lacked sufficient protoscolex and for which DNA extraction was unsuccessful during the preliminary study (Table 1). PCR was used to analyze the ribosomal ITS-1 gene region of 13 [paraffinized] human [11.5%] and 47 sheep [41.60%] isolates from a total of 60 samples. 40 of these isolates were found to share the same band sequence (Figure 1).

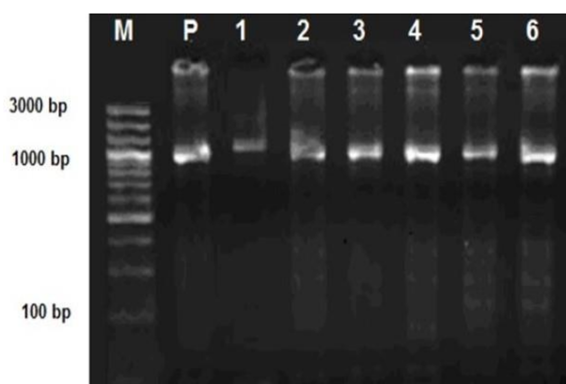

Figure 1. The appearance of the bands formed as a result of PCR amplification of the ITS-1 region of the sheep and human DNA products of *E. granulosus*. M: Deoxyribonucleic acid size marker (100 bp); P: Reference G1 sheep strain 1,2,3: Sheep isolate; 4,5,6: Human isolate.

197 samples were examined in the study. Of these, 113 were fertile [57.36%] and 84 were sterile. In 113 fertile samples, a total of 60 isolates remained after removing hydatid cyst materials that lacked sufficient protoscolex and for which DNA extraction was unsuccessful during the preliminary study (Table 1).

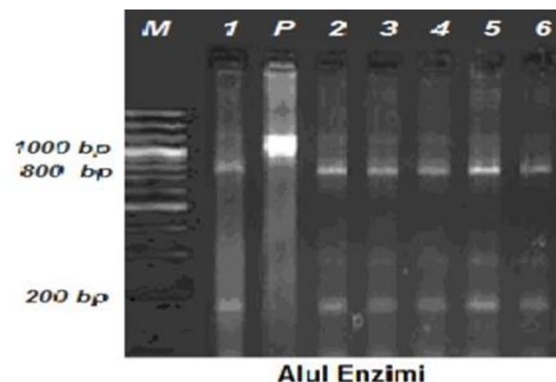

Figure 2. Ethidium bromide-stained agarose gel image of ITS-1 PCR products cut with AluI. M: Deoxyribonucleic acid size marker (100 bp); P: PCR product present, no restriction enzyme 1, 2, 3: Sheep isolate; 4, 5, 6: Human isolate.

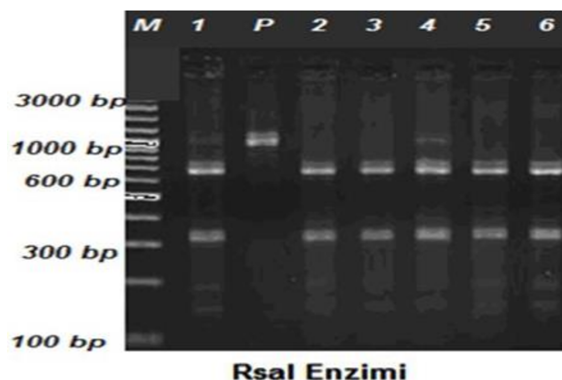

Figure 3. Ethidium bromide-stained agarose gel image of ITS-1 PCR products cut with RsaI. M: Deoxyribonucleic acid size marker (100 bp); P: PCR product present, no restriction enzyme 1,2,3: Sheep isolate; 4,5,6: Human isolate.

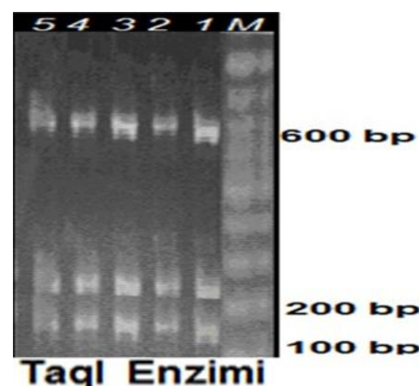

Figure 4. Ethidium bromide-stained agarose gel image of ITS-1 PCR products cut with TaqI. M: Deoxyribonucleic acid size marker (100 bp); 1, 2, 3: Sheep isolate; 4.5: Human isolate.

When comparing these band profiles to the reference G1 [sheep strain-G1], it was determined that they were all the same; hence, the isolates included in the study were sheep strains [G1] (Figure2, Figure3, Figure4). The band profiles shown in the figures were obtained with the cutting process of ITS-1 positive PCR products with AluI, RsaI, and TaqI enzymes (Çalışkan, 2015).

Hydatidosis is a zoonotic illness that remains significant in many regions of the world including Türkiye due to the economic losses it generates and its impact on human and animal health. It is a globally prevalent and significant helminth zoonosis. There are five kinds in the genus *Echinococcus* with *E. granulosus* being the most important and prevalent, and there are significant strain differences within the kinds. The definitive host of each kind is a carnivore, while the middle host may be one of many mammalian species. Hydatid cysts are more prevalent in Southeastern Anatolia, Central Anatolia, Marmara, and Thrace regions in Türkiye. Numerous studies have used PCR-RFLP and DNA sequence analysis to identify *Echinococcus* strains in various geographical regions of the world (Hokelek & Arıkoğlu, 2004; Lavikainen et al., 2003; Thompson & McManus, 2002; Unat et al., 1995; Yildiran et al., 2009; Yolasığmaz & Altıntaş, 2004; Borhani et al., 2022).

Molecular characterization of *E. granulosus* was studied by the PCR-RFLP method and the *E. granulosus sensu stricto* [G1-3 complex] strain was found to be the dominant genotype on farm animals in Iran (Fallahizadeh et al., 2019). In another study in Iran, sequence analysis of the cox-1 and nad-1 genes of 44 paraffin-embedded human hydatid cyst tissues revealed that 86.3% of the samples had G1 strains and 13.6% contained G6 strains. It was thought that the sheep [G1] and camel [G6] strains were crucial for transmission (Mirahmadi et al., 2021). In a molecular study conducted in Uzbekistan on 52 human and 6 sheep hydatid cyst samples and 10 adult echinococcus samples simultaneously collected from dogs, G1 and G3 strains were identified as the predominant genes (Kim et al., 2020).

Diverse biological and morphological criteria are taken into account in the differentiation of strains, and the role of molecular approaches for precision is increasing rapidly. Strains of *E. granulosus* are often identified and categorized by PCR-based techniques. Both parasite genome research and diagnostic molecular studies rely on PCR, RFLP, PCR-RFLP, RAPD-PCR, SSCP, dideoxy fingerprinting [ddF], and DNA base sequence analysis (Hokelek & Arıkoğlu, 2004; Örsten, 2017; Unat et al., 1995; Vural & Muz, 2017; Yildiran et al., 2009).

In a work by Vural et al. the sequence analysis of the CO1 gene zone of *E. granulosus* isolates from 100 sheep and 12 cattle from Afyon, Ardahan, Erzurum, Siirt, Tekirdağ, Yozgat, and Kars provinces revealed that 107 of the samples were categorized as G1 and 5 of them were as G3, respectively (Vural & Muz, 2017). Utuk investigated the molecular identification of *E. granulosus* isolates recovered from 19 cattle, 179 sheep, 1 camel, 7 goats, 1 human, and 1 dog from Elazığ, Erzurum, Malatya, Diyarbakır, Van, and Şanlıurfa. Cattle, goat, and sheep isolates were researched by PCR-RFLP methods handling, restriction enzymes CfoI, MspI, RsaI, and AluI (Utuk, 2008). It was noted that all isolates exhibited similar patterns. The sequence analysis of the mitochondrial CO1 gene region of randomly selected isolates from 6 cattle, 4 sheep, 4 goats, 1 camel, 1 dog, and 1 human isolate revealed 17 isolates as the G1 strain of *E. granulosus.* Using the size and sequence of the nuclear genomic rDNA-ITS1 zone, the PCR-RFLP method enables easy and rapid separation of *Echinococcus* isolates.

Ribosomal RNA [rRNA] genes are organized into rDNA units with highly conserved coding zones and relatively weakly conserved non-coding gap regions between these regions. It is assumed that the restriction enzyme, which cuts every four bases, cuts the approximately 1 kb-long ITS1 region four times. In other words, each enzyme is expected to examine 16 nucleotides (Keyhani et al., 2020). In this study, the rDNA ITS-1 gene sections of 13 human and 47 sheep isolates were investigated by PCR-RFLP method utilizing the restriction enzymes Rsal, Alul, and Taql, which have been used in numerous other studies (Lavikainen et al., 2003; Mirahmadi et al., 2021; Utuk, 2008; Vural & Muz, 2017; Yolasığmaz & Altıntaş, 2004). If possible, working with a fresh sample makes it easier to carry out the procedure. Because it is very difficult to remove paraffin from the sample. As a result of the analysis, it was stabled that our isolates were identical to the G1 reference strain. Baş, Y. et al. also revealed similar results (Baş et al., 2021). The results of this work are consistent with works indicating that the domestic sheep strain of *E. granulosus* is the dominant genotype in Türkiye (Acıöz et al., 2018; Keyhani et al., 2020; Lavikainen et al., 2003; Örsten, 2017; Utuk, 2008; Yolasığmaz & Altıntaş, 2004). Hydatid cysts continue to be a problem in the fight against CE due to the high number of stray dogs, the fact that butchery animals are not slaughtered under the rules, especially in rural areas, our people are not educated about the disease, and many other factors (Yazar, 2002). The data from the Ministry of Health shows that there has been an increase in the number of reported cases over the years. The number of 408 cases in 2008 reached up to 1,867 in 2019 (Erdoğan et al., 2017).

It can be observed that the difference in the number of cases, especially in the years 2016-2017, is at the highest rate. Few field research on the epidemiology of CE exists in Türkiye. The literature study reveals that additional attempts have been made to conduct seroepidemiological, pathological, or radiological research; nevertheless, these studies have only been conducted in a few regions (Türkoğlu et al., 2017; Vural & Muz, 2017; Yılmaz et al., 2016).

In regions where a species is endemic, molecular methods allow for accurate species distinction. As the World Health Organization Echinococcosis study group stated in the guide published in 2010, there is no gold standard treatment for CE (Altintas et al., 2021; Erdoğan et al., 2017; Türkoğlu et al., 2017). Considering all these

factors, avoiding sickness is considerably simpler and more vital than receiving treatment. Controlling the dogs, which are the final hosts and have a complex relationship with humans, maybe the simplest method for breaking this cycle. For this reason, all dogs should be registered, and a stray dog collection area should be established. Dogs should not be permitted in places where people congregate, such as schools, shopping centers, and public transportation vehicles (Alemu et al., 2015; Gonzalez & Gomez-Puerta, 2018; Kern et al., 2017; Şenlik, 2013). Veterinarians should examine dogs, and if necessary, treatment should be organized. In addition, dogs should not consume organs with hydatid cysts. For this reason, stray slaughter should be severely restricted and not permitted. The slaughterhouses should be located outside the city limits, and dogs should not be permitted to roam the city. Organs from slaughtered animals should not be discarded publicly, nor should they be fed to dogs. Dogs should not be fed raw or organ meats (Gonzalez & Gomez-Puerta, 2018). Control methods developed in the 1860s to lessen the spread of *E. granulosus* from sheep to dogs are still effective today (Craig et al., 2017).

CONCLUSION AND SUGGESTIONS

In this study, hydatid cyst material taken from various intermediate hosts of *E. granulosus* was analyzed by PCR-RFLP, and the genetic diversity of the province of Şanlıurfa was researched. As an effect of the research, it was determined that the fertility rate of hydatid cysts in the sheep in this study was significantly higher than in the cattle, and it was determined for the first time that the common genotype was the domestic sheep strain with different strains specific to Şanlıurfa province. This determination serves as a reference for the scope and method of measures to be performed in this region to manage CE, a serious public health issue. It would be appropriate to conduct extensive epidemiological/molecular research at regular intervals and to establish eradication initiatives to manage CE in Türkiye.

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Statement of Research and Publication Ethics

Ethical approval for this study was obtained from the Harran University Medical School Ethical Board on 17.03.2014 with the number 74059997.050.01.04\039.

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