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Original Article

Investigation of Mitochondrial *Cytb* Gene Region of Both *Echinococcus granulosus* Eggs from Dogs and Cystic Echinococcosis Isolates Obtained from Sheep and Cattle by Molecular Methods

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Abstract

Background: We aimed to determine the common *Echinococcus granulosus* genotypes in Ağrı, Türkiye and to obtain information on the transmission of this parasite.

Methods: Cystic echinococcosis samples from 100 slaughtered cattle and 100 slaughtered sheep and faecal samples from 200 stray dogs were included in 2021. Collected cyst fluid samples and faces were examined microscopically. DNA was isolated from the germinal membrane of the cysts and from the parasite eggs in the stool samples. The mitochondrial *cytb* gene region of the parasite was amplified by PCR. Genotypes were determined using the Basic Local Alignment Search Tool (BLAST) after sequence analysis of PCR amplicons.

Results: The highest percentage of cysts was found in the lungs of sheep and the liver of cattle. In addition, 75% of sheep cysts and 25.6% of cattle cysts were fertile. Taenia spp./*Echinococcus* spp. eggs were found in 6% of the faeces of 200 dogs examined microscopically. *E. granulosus* eggs were detected in 4 out of 50 stool samples analysed by PCR. All samples analysed by sequence analysis were identified as *E. granulosus* s.s. G1 genotype. Sequence comparison revealed revealed one or more-point mutations in different regions of the five samples.

Conclusion: *E. granulosus* s.s. G1 genotype, known as sheep strain, is common in the Ağrı, Türkiye. The controlled slaughter of livestock, especially sheep, and the avoidance of feeding hydatid cyst organs to dogs, together with public education, were necessary to prevent the spread of the disease.

Introduction

Cystic echinococcosis (CE) is an important zoonotic parasitic disease caused by different species of the genus *Echinococcus*, which is widespread in Türkiye and worldwide, causing death and great economic loss to humans and animals. (1). The final hosts of the parasite are carnivores such as foxes, wolves, and jackals, especially dogs. Parasites that live in the intestinal lumen of the final hosts are excreted into the environment with feces by the disintegration of the ring or rings. Eggs taken by intermediate hosts such as ruminants and, rarely, humans, are broken down by enzymes in the stomach and small intestines, causing the oncosphere to be released. The released oncosphere adheres to the intestinal lumen by hooks and reaches the lamina propria in the villus epithelium. From here, they pass through the venules and reach the liver and lungs, rarely to brain, muscle, kidney, spleen and other organs, and form fluid-filled cysts (1, 2).

In developed countries, the spread of CE has decreased due to the high educational level of the people and the regular treatment of dogs with anti-parasitic drugs (3). However, in developing countries, factors such as not paying attention to hygiene rules, uncontrolled and illegal slaughter of animals, the high number of stray dogs, and the indiscriminate release of cystic organs into the environment crucial an important role in the spread of the parasite. Factors such as the extensive agriculture and animal husbandry in Türkiye, the low socioeconomic level especially in rural areas, the favourable climatic conditions for the spread of the parasite, and the existence of uncontrolled and illegal slaughterhouse increase the incidence of CE (4).

With the widespread use of molecular methods, 10 different genotypes from G1 to G10 were determined within the *E. granulosus* species. However, in the most recent molecular studies, the parasite known as *E. granulosus* is classified under five (4) different species. These are *E. granulosus* sensu stricto (s.s.) (G1, G2 and G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6, G7, G8 and G10) and *Echinococcus felidis* species. However, because of recent taxonomic studies, G2 strain was accepted as a micro variant of G3 strain. It has been reported that *E. granulosus* s.s. is the most common strain among these five species and is most responsible for the development of the disease in humans (5-7).

The aim of this study was to determine the common genotypes of *E. granulosus* in Ağrı Province, to obtain information on the transmission of this parasite and to determine the mutations that occur.

Materials and Methods

Ethical approval for this study was obtained from Van Yüzüncü Yıl University (Approval Number: 2020/12-10).

Various slaughterhouses operating in Ağrı, Türkiye were visited at regular intervals in 2021. CE samples were taken from 100 sheep and 100 cattle. Stool samples were collected during defecation from 200 stray dogs. To avoid contamination, samples were taken from the upper part of the faeces of dogs observed defecating. All the samples were kept in sterile bags and were delivered to the research laboratory of the Department of Parasitology, Faculty of Medicine, Van Yüzüncü Yıl University. By paying attention to the protection and safety conditions, the germinal membranes and the samples from the bottom sediment were taken from the cysts and they were placed in 1.5 ml eppendorf tubes. Cyst and stool samples were placed in a deep freezer set at -20°C to be studied later.

Microscopic examination of CE fluids

In order to evaluate the cyst fluid samples in terms of fertility, the samples were centrifuged

at 1000 rpm for 5 minutes. The slides were prepared from the bottom part of the residue and were examined under the light microscope (Leica DM500) with a 40X objective for the presence of protoscolex or hook. Specimens with protoscolex or hook were considered as fertile cysts.

Examination of Stool Samples

Stool samples were first examined macroscopically for the presence of cestode rings under sufficient light. Then, flotation method was applied to detect the presence of helminth eggs in the samples (8).

DNA isolation from cyst and stool samples

DNA was isolated from the germinal membrane of 20 fertile cysts, 10 from sheep and 10 from cattle. In order to isolate DNA from CE samples, 4-5 grams of germinal membrane were taken and fragmented with the help of a homogenizer. DNA isolation from the germinal membrane was performed using the Genomic DNA Purification Kit (Invitrogen K1820-20) in accordance with the manufacturer's recommendation.

DNA extraction was performed using the stool DNA purification kit (EURX E3575) from a total of 50 faecal samples, 12 of which *Taenia* spp. / *Echinococcus* spp. eggs were detected in microscopic examination of faeces and 38 randomly selected from negative faeces. Before starting DNA isolation, 5-10 grams of faecal samples taken in Eppendorf tubes were kept in liquid nitrogen tank (-196°C) for 1 min and then in 90°C hot water for 5 min with 3 repetitions. Afterwards, the manufacturer's instructions were applied.

PCR amplification of the mitochondrial cytb gene region

CYTBF 5' AGATATTAGAGATGGAGGAG 3' and CYTBR 5' ACCGAGTTAATACAGTCAG 3' primers were used to amplify the *cytb* gene region (9). The reaction was adjusted to a total volume of 40 µL, containing 8 µL of Taq 5x Master Mix (with 12.5 mM MgCl2) (Thermo Fisher), 0.5 mM of MgCl2, 0.2 µM from each primer, and 2.5 µL of sample DNA.

PCR was carried out on a SimpliAmp Thermal Cycler (Applied Biosystems). PCR was programmed for 45 cycles of 30 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.

Sequence analysis

The PCR product of the mitochondrial *cytb* gene region was sent to Sentegen Biotech Company (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.

Table 1: GenBank numbers and citations of the reference sequences used in the study

Genotype	*Genbank number*	*Reference*
G1	AF297617.1	Zhong et al. 2014 (9)
	KJ556992.1	Khan et al. 2020 (13)
	KJ556990.1	Khan et al. 2020 (13)
	KJ556991.1	Khan et al. 2020 (13)

Results

The distribution of organ location and the number of caseifications of cyst samples are given in Table 2. Because of microscopic examination of non-caseating cyst fluids, fertility was detected in a total of 84 cyst samples, including 63 (75%) of 84 sheep cysts and 21 (25.6%) of 82 cattle cysts (Fig. 1) (Table 3).

Table 2: Organ location and number of caseified cysts in CE samples

Host	*Organ*	*The number of non-caseating cysts (%)*	*The number of caseified cysts (%)*
Sheep	Lung	32 (80.0)	7 (20.0)
	Liver	29 (76.3)	9 (23.7)
	Lung + Liver	21 (100.0)	-
	Spleen	1 (100.0)	-
	Kidney	1 (100.0)	-
	Total	84 (84.0)	16 (16.0)
Cattle	Lung	23 (88.5)	3 (11.5)
	Liver	37 (71.2)	15 (28.8)
	Lung + Liver	18 (100.0)	-
	Heart	1 (100.0)	-
	Spleen	2 (100.0)	-
	Kidney	1 (100.0)	-
	Total	82 (82.0)	18 (18.0)
Total		166 (83.0)	34 (17.0)

Table 3: Fertility distribution of cyst samples according to hosts

Host	*The number of fertile cyst (%)*	*The number of sterile cyst (%)*	*Total number of cysts examined.*
Sheep	63 (75)	21 (25)	84
Cattle	21 (25.6)	61 (74.4)	82

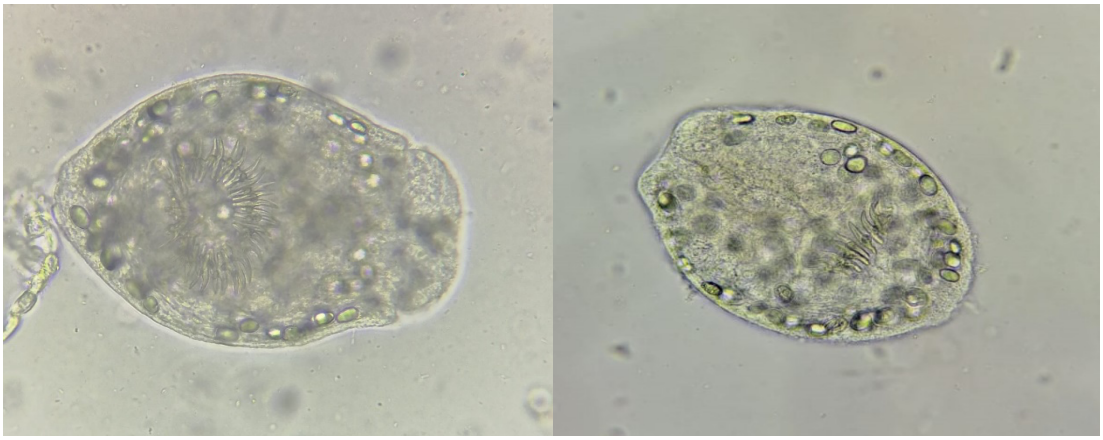

Fig. 1: Protoscolex detected in the cyst fluid sample examined under the microscope (Original)

Light microscopy of 200 dog faecal samples revealed the presence of Taenia spp./*Echinococcus* spp. eggs in 12 (%6) of the samples. Three of 12 microscopically positive samples and one of 38 randomly selected samples were positive by PCR (Fig. 2).

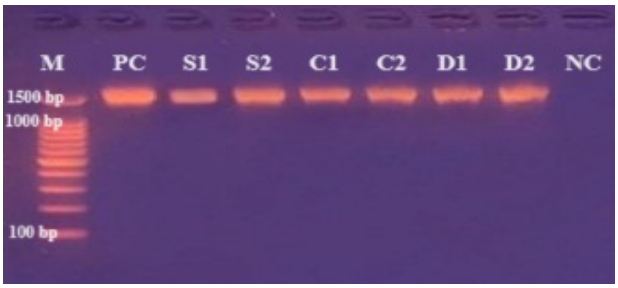

Fig. 2: Results of PCR amplification of the mitochondrial cytb gene region (1609bp) of cyst samples; M: 100 bp DNA marker (Thermo Mark), PC: Positive control, NC: Negative control, S1, S2: Sheep samples, C1, C2: Cattle samples, D1, D2: Dog samples

DNA Sequence Analysis Results of Mitochondrial cytb Gene Region

When the sequence results were analysed in SnapGene software programme, it was determined that the cytb gene region with a length of 1070 bp was error-free for all of the samples. After performing BLAST, all of the samples were determined to be a *E. granulosus* s.s. G1 genotype. When the 24 samples were compared with the reference G1 genotype in Genbank, eight sheep, seven cattle and four dog samples matched completely with the reference G1 genotype (AF297617.1), and one or more-point mutations were found in the remaining three cattle and two sheep samples (S1,2,3,4,5). Mutation-detected sequences were registered to the NCBI with accession numbers; PP464293, PP464294 for sheep and OR933694, OR933695, and OR933696 for cattle.

Discussion

Recently, molecular techniques such as PCR, RFLP, PCR-RFLP, RAPD-PCR, SSCP, and DNA sequencing are used to determine the genetic diversity within the genus *Echinococcus*. However, DNA sequencing method is accepted as the gold standard among these molecular techniques. It minimizes errors because of the analysis of the double DNA chain. In addition to the advantages of the method such as reliability and high discrimination power, the high cost of the method is a disadvantage (10).

Since genotype variation in *E. granulosus* varies in host specificity and transmission dynamics, genotyping CE cases plays an important role in the formulation of control strategies to prevent transmission of this parasite (11). As a result of molecular studies on different gene regions in cyst samples in humans, it has been reported that *E. granulosus* s.s. G1-G3 genotype is common (6, 10-18). In studies carried out with farm animals, G1 and G3 genotype were more prominent, hovewer different genotype were also determined (7,15,17,19-24). As a result of molecular studies on the adult form or egg of the parasite in dog feces, the G1 strain was generally detected and it was reported that other genotype were also found (16, 25-27). In Türkiye, different genotypes have been detected in farm animals, but the G1 genotype has been identified as the dominant strain (1, 28-31). Similarly, the G1 genotype was found to be the dominant strain in dogs in Türkiye (32-34). In this study, all of sheep, cattle and dog isolates were G1 genotype. The fact that the G1 genotype, known as the sheep strain (11), was detected in all iso-

lates indicates that sheep play a role in the prevalence of CE in the Ağrı, Türkiye.

The G1 genotype, commonly known as the sheep strain, predominantly targets sheep. Although it can infect other intermediate hosts such as cattle, goats, and dogs, the fertility rate of cysts in these animals is either low or absent (11). In this study, it was found that the fertility rate of the cysts, which was 75% in sheep, decreased to 25.6% in cattle. The low fertility of the G1 genotype in livestock other than sheep may facilitate eradication studies. We believe that only the G1 genotype was detected in the Ağrı region in this study shows eradication is possible. The first step towards eradication in the Ağrı region is to prevent dogs from accessing the sheep organs affected by CE.

Numerous studies have been carried out in Türkiye to determine different species and genotype of *Echinococcus* and CE, as well as mutations. In a study conducted in Elazığ, mutations in the mt-CO1 gene region were detected in 16 out of 66 cattle isolates and 7 out of 19 sheep isolates (29). In a study in which samples were collected from Elazığ, Bingöl and Erzincan, a total of 49-point mutations were detected in the mt-CO1 gene region of 60 cattle CE isolates (30). Point mutation was detected in four of 38 human CE isolates in Van province (11). In this study, one or more-point mutations were found in three samples from cattle and two samples from sheep.

Conclusion

The fact that only the G1 strain is found in the region shows that sheep play an important role in the prevalence of CE in Ağrı province. As the fertility rate of cysts in G1 strain sheep is high, sheep should not be ignored in eradication studies. Since the most important ways to prevent this disease is not to allow fertile cysts to be consumed raw by the last hosts, to treat infected last hosts, and to raise awareness of the public about CE, we believe that action should be taken within the scope of these substances.

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Data availability

Supplementary files will be sent to applicants in case of reasonable apply by the corresponding author.

Conflict of Interest

Non-declared.

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