

Investigation of *Echinococcus granulosus* in Stray Dogs by Real Time PCR in Izmir Province, Turkey

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ABSTRACT

Objective: The aim of the study is to determine the prevalence of *Echinococcus granulosus* sensu lato by molecular analysis with Real Time PCR (RT-PCR) method by collecting stool samples from stray animals that can be reached in 9 of the 10 determined districts of Izmir province (Aliaga, Menemen, Bornova, Urla, Selcuk, Bayindir, Odemis, Tire, Kiraz).

Materials and Methods: Thirty samples from Aliaga, 41 samples from Menemen, 35 samples from Bornova, 10 samples from Urla, 40 samples from Selcuk, 40 samples from Bayindir, 33 samples from Odemis, 45 samples from Tire, 26 samples from Kiraz, totally 300 stool samples were collected. The samples were left at -80°C for 5 days for inactivate. Afterwards, all inactivated stool samples were kept at -20°C until they were taken into the study. DNA isolation from stool samples was carried out with DNA extraction kit. The purity of all isolated samples was measured by spectrophotometer. The analysis of the RT-PCR results was performed with the Rotor-Gene Q series software 2.3.1 program and the samples were evaluated for *E. granulosus* s.l. positive/negativeness.

Results: According to the results of RT-PCR analysis, *E. granulosus* s.l. positive sample was not found in stool samples collected from stray dogs in the study area.

Conclusion: Since no molecular studies have been carried out in the districts included in our study, the results obtained could not be compared. It also can be thought that the number of samples is insufficient. Therefore, a larger sample pool should be examined to find out the prevalence of *E. granulosus* s.l. Since there is limited data about the prevalence of *E. granulosus* s.l. in stray dogs, it also is important to investigate the prevalence of *E. granulosus* s.l. in different regions of Turkey.

Keywords: Cystic echinococcosis, *Echinococcus granulosus*, DNA, Real Time PCR

INTRODUCTION

Cystic echinococcosis (CE) is a chronic parasitic zoonosis caused by the larval stage of the cestode *Echinococcus granulosus* sensu lato (s. l.) (1,2). CE, which is very common in the world, affecting humans, domestic animals and wild mammals, causes significant economic losses in both the medical and livestock sectors (1,3,4). This disease, which threatens health by causing significant morbidity and mortality in humans, is on the list of 17 neglected tropical diseases, as well as on the list of priority neglected zoonotic diseases that are aimed to control or eradicate (World Health Organization (WHO) 2017) (4,5).

In the life cycle of *E. granulosus* s.l., the adult form of the parasite requires dogs and other canines as definitive hosts, while the larval form requires herbivores or omnivores as intermediate hosts. Adult *Echinococcus* spp. live in the small intestine of the canids. Humans are accidental intermediate hosts in this cycle, infected by accidental ingestion of eggs released in canid stool (3,5,6).

Common on all continents, including circumpolar, temperate, subtropical, and tropical regions, CE is responsible for 95% of human cases of echinococcosis and has a worldwide prevalence of approximately six million (2,3).

CE is commonly seen in livestock areas in South America, North Africa, Australia, western, central and eastern Europe and central Asia, particularly western China. Globally, at least 50 million people are infected with *E. granulosus*, with approximately more than 170,000 new cases each year. The estimated minimum global human burden is a public health problem that reaches 285,500 disability-adjusted life years (DALYs), resulting in economic losses of approximately US\$3 billion annually (5,7). According to the data of the Ministry of Health between 1990 and 2005 in Turkey, 52.124 cases were operated for CE. Recently published an article by Ministry of Health reported that the number of cases was 408 in 2008 and reached 1.867 at the end of 2019. According to other research data, the estimated case incidence was published as 0.8-2 per 100,000 or 0.3-0.087%. According to limited local study data from different geographical regions, the prevalence of *E. granulosus* infection in Turkey is between 0.32% and 40% in dogs. When the current status of echinococcosis in the dogs is evaluated with the PCR technique, which is widely used in recent years, it varies between 4.0-14.0% (8,9,10).

Molecular analyzes were performed in *Echinococcus* spp. using methods such as Restriction Fragment Length Polymorphism (RFLP), Multiplex-PCR, High Resolution Melting analysis (HRM), Loop-Mediated Isothermal Amplification (LAMP), and PCR gene amplification followed by sequencing. Mitochondrial gene loci such

as cytochrome c oxidase subunit 1 (cox1) and NADH dehydrogenase subunit 1 (ND1) are frequently used in analyzes (1).

With the molecular-based studies carried out to date, *E. granulosus* s.l. species cluster has been tried to be clarified. As a result, with phenotypic differences in morphology and biology, eight different genotypes based on genetic differences in mitochondrial genes has been clarified; *E. granulosus* sensu stricto (s.s.) (genotypes G1 and G3), *E. equinus* (genotype G4), *E. ortleppi* (genotype G5), *E. canadensis* cluster (genotypes G6-8 and G10) and *E. felidis* (6,11).

In Turkey, there is limited data on the prevalence of *E. granulosus* s.l. on the definitive hosts. Therefore, the main goal of the present study is to determine the prevalence of CE with Real Time PCR (RT-PCR) method with using mitochondrial ND1 gene for characterize *E. granulosus* isolates, by collecting stool samples from stray animals that can be reached in 9 of the 10 determined districts of Izmir province.

MATERIALS AND METHODS

Material Collection

Turkish Association of Hydatidology, with "Creating Awareness of Cystic Echinococcosis in Izmir Province" project the "Local NGOs Grant Program" carried out under the Civil Society Support Program were entitled to receive grant support by the European Union and the Republic of Turkey under the Instrument for Pre Accession Assistance (IPA II) 2014 program. This project was carried out between 2019-2020 years. Stool samples collection, from stray animals, for molecular studies was carried out in 9 of the 10 districts of Izmir selected for the project, which have dog shelters (Aliaga, Menemen, Bornova, Urla, Selcuk, Bayindir, Odemis, Tire, Kiraz). Dog stool collection and storage procedure, stool collection containers (Isolab) were given to the veterinarians working in the dog shelters. Veterinarians took stool samples, according to stool collection procedure, before parasite treatment when dogs came to the dog shelter. From the dogs brought to the shelter from the districts determined within the scope of the project, 5-10 g stool samples were taken into the stool storage containers by the shelter veterinarian. Samples were kept at -20°C until the project team received them. Each sample was labeled with the ear tag number of the animal it belongs to, the date and the district it was taken from. Stool samples were delivered to the laboratory in a cold chain box as soon as possible.

Stool samples, after reaching the laboratory, labeling according to the district that collected, was performed at Ege University, Echinococcosis Research Laboratory. Stool samples were kept at -80 °C for 5 days for inactivation. The

samples were then stored at -20°C until use. Totally 300 samples were collected from selected 9 districts during the Project. The sample numbers collected by districts are shown on the map (Figure1).

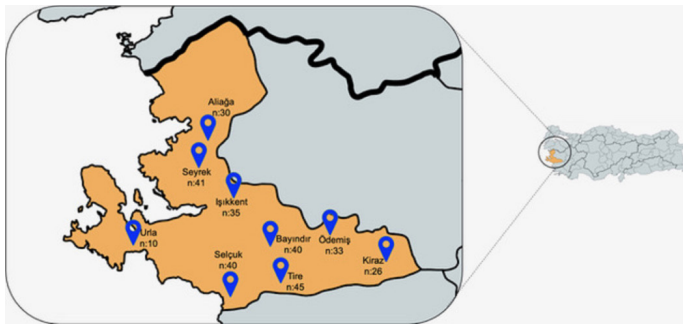


Figure 1. Map of samples which was collected from districts

Molecular Analysis

DNA isolation from stool samples was done with commercial kit (Qiagen, Qiamp Fast DNA Stool Mini Kit) according to the manufacturer's instructions. An average of 200 mg of stool samples was used for isolation. DNA isolations were performed according to the manufacturer's protocol; Lysis of and separation of impurities from stool samples in InhibitEX Buffer, Purification of DNA on QIAamp Mini spin columns. DNA isolation steps also was performed at Ege University, Faculty of Medicine, Department of Parasitology, Echinococcosis Research Laboratory. The purity measurement of the isolated DNA was performed with Nanodrop spectrophotometer (Thermo Scientific). The purity of extracted DNA was measured by A260/A280 nm ratio. Samples of suitable purity ($\geq 1,8$) were included in the study. Purified DNA's from each sample was diluted approximately to 5 ng/ μ l, aliquoted and stored at -20°C until RT-PCR application. Mitochondrial ND1 gene used to characterize animal *E. granulosus* isolates with molecular techniques, was used also in our study.

In RT-PCR method, we used ND1 gene *E. granulosus* specific primer/probe mix (*E. granulosus*, NADH dehydrogenase subunit 1 gene, genesig Advanced Kit, Primer Design™ Ltd.) ready-to-use, optimized by the manufacturer. We used also 2X qPCR Master Mix (oasig™ lyophilised 2X qPCR Master Mix, Primer Design™ Ltd.), ready-to-use, optimized by the manufacturer. RT-PCR protocol also was prepared according to the protocol; Enzyme activation 95°C 2min, Cycling X 50: Denaturation 95°C 60 sec, Data Collection 60°C 10 sec. All samples were run on a Rotor Gene Corbett RT-PCR (Qiagen) instrument for amplification. RT-PCR steps was performed at Manisa Celal Bayar University, Faculty of Medicine, Department of Medical Biology, Molecular Biology Laboratory.

RESULTS

The threshold value (basing 0.3) of the obtained RT-PCR results was checked for positivity or negativity using the Rotor-Gene Q series software 2.3.1 program. Values above the threshold value were considered as positive, while the value below was considered as negative. The ND1 gene amplification curves of stool samples were observed in RT-PCR (Figure 2). Molecular analysis results of stool samples taken from dog shelters showed that *E. granulosus* s.l. agent was not found because all samples were below the threshold value, and the dogs in our study group were not infected with *E. granulosus* s.l.

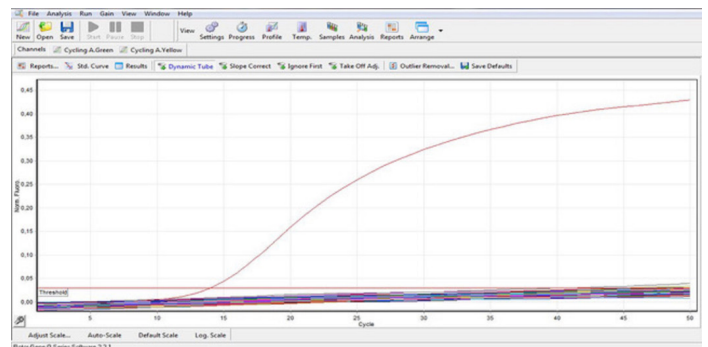


Figure 2. ND1 gene amplification curves of stool samples in Real Time PCR

DISCUSSION

Aim of this study was investigation of the distribution of *E. granulosus* s.l. in stray dogs based on stool samples collected from 9 of the 10 selected districts of İzmir, Turkey. It presents for the first data from this districts on *E. granulosus* s.l. prevalence from stray dogs.

Although dogs infected with *E. granulosus* s.l., which has a worldwide zoonotic and epidemiological distribution, are the main source of CE, little is known about the regional molecular epidemiology of adult *Echinococcus* spp. in stray dogs. With research to determine the presence and genotype frequencies of CE in definitive host populations, control programs can be established and progressed, while also assessing its distribution, host specificity, transmission dynamics, and risk of infection for humans in a given area (12). In the region where we collected samples for our study, no molecular study has been done before in stray dogs.

The epidemiology of echinococcosis has made significant progress based on molecular methods in the last ten years (13). In recent studies, serological and molecular analyzes of dog stool have been carried out in addition to traditional methods. PCR, which is one of the molecular methods; since it is an easily applicable, highly sensitive, specific method using closed tube systems, it offers the advantages

of being able to scan a large number of samples in a short time, reducing the risk of contamination. Because of these advantages, it is frequently used in studies to determine the prevalence of *E. granulosus* from dog stool (14). Determining the prevalence of *E. granulosus* from dogs by molecular methods has been used in different parts of the world. PCR, PCR-RFLP, copro PCR, multiplex PCR and sequence analysis was done from necropsy or stool material of stray, domestic and bred dogs (12,13,15-19). In our study, RT-PCR method was performed to determine the prevalence of *E. granulosus* s.l. in stray dogs from the collected stool materials in 9 districts of Izmir province.

In studies of the molecular identification of *E. granulosus* spp., two mitochondrial genes, ND1 and cox1 were most frequently used (13,15-19). In our study, also the ND1 gene was used for molecular identification.

In the world, between 2009 and 2019, studies were conducted on the prevalence of *E. granulosus* in dogs using PCR, Copro-Antigen ELISA and necropsy methods. As a result of these studies, the prevalence of *E. granulosus* varies between 1.1-10.6% in Europe, 12.2-51.2% in the Middle East and Africa, 3.15-50.7% in Asia, Far East and Oceania, and 0.4-36% in the USA (10). In different regions of Turkey, *E. granulosus* in dogs was mostly screened by necropsy method and the prevalence was found between 0.94-54.5% (14). And also the prevalence of *E. granulosus* in dogs was reported between 0.8-10.8% by PCR and stool examination methods (10). In our study, we couldn't find any positivity from collected samples by RT-PCR method. This result which is found in this study is different than the other studies conducted in different parts of Turkey. According to the veterinarians who were collected the samples from these stray dogs, the reason for the absence of the agent in stool samples collected from animal shelters may be that dogs are fed with dog food in dog shelters.

CONCLUSION

Since no molecular studies have been carried out in the districts included in our study, the results obtained could not be compared. It also can be thought that the number of samples should be insufficient. Therefore, a larger sample pool should be needed to find out the prevalence of *E. granulosus* s.l.. Except for the countries and regions where control studies have been carried out successfully, CE remains an important parasitic disease and socio-economic problem for humans and animals in many parts of the world as well as in Turkey. In countries where economy based on livestock like Turkey, it's so important to prevent the sheep-dog cycle. Unfortunately there is no accurate data and the map of distribution of *E. granulosus* in Turkey. As a result; CE urgently needs attention both for protecting public health and animal welfare. Control of CE can be taken under control if it is given the priority and if

the necessary measures are taken by the authorities. So, serious control studies should be carried out against CE as soon as possible.

Competing interests: The authors declare that they have no competing interest.

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Ethical approval: This study did not involve any experimentation with live animals.

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REFERENCES

1. Avila HG, Santos GB, Cucher MA. et al. Implementation of new tools in molecular epidemiology studies of *Echinococcus granulosus* sensu lato in South America. *Parasitol Int.* 2017;66: 250–7.
2. Hammad SJ, Cavallero S, Milardi GL, et al. Molecular genotyping of *Echinococcus granulosus* in the North of Iraq. *Vet Parasitol.* 2018;249:82–7.
3. Ozkeklikci A, Akdur Ozturk E, Akil M, et al. Investigation of patients with suspected cystic echinococcosis by ELISA and IHA Methods in Gaziantep, Turkey. *Int J Echinococcoses.* 2022;1:1-6.
4. Altintas Nu., Oztatlici M., Altintas Na. et al. Molecular analysis of cattle isolates of *Echinococcus granulosus* in Manisa province of Turkey. *Kafkas Univ Vet Fak Derg* 2013;19:55-9.
5. Kurt A, Guven E, Balkaya I, et al. Molecular characterization of echinococcus multilocularis and echinococcus granulosus from cysts and formalin-fixed paraffin-embedded tissue samples of human isolates in northeastern Turkey. *Vector-Borne Zoonotic Dis.* 2020;20:8.
6. Soba B, Gaspersic S, Kese D, et al. Molecular Characterization of *Echinococcus granulosus* sensu lato from Humans in Slovenia. *Pathogens* 2020; 9: 562.
7. Xin Q, Yuan M, Lv W, et al. Molecular characterization and serodiagnostic potential of *Echinococcus granulosus* hexokinase. *Parasites Vectors.* 2021;14:105.
8. Deplazes P, Rinaldi L, Alvares Rojas CA, et al. Global distribution of alveolar and cystic echinococcosis. *advances in parasitology* 2017;95:ISSN 0065-308X.

9. Altintas N. Parasitic zoonotic diseases in Turkey. *Vet Italiana*. 2008;44:633-46.
10. Altintas N, Topluoglu S, Yildirim A, et al. Current situation report of cystic echinococcosis in Turkey. *Turkish Bulletin Hygiene Experimental Biol*. 2020;77:1-51.
11. AL-Mutairi NM, Taha HA, Nigm AH. Molecular characterization of *Echinococcus granulosus* in livestock of Al-Madinah (Saudi Arabia). *J Helminthol*. 2020;94:1-12.
12. Ghabdian S, Borji H, Naghibi A. Molecular identification of *Echinococcus granulosus* strain in stray dogs from Northeastern Iran. *Vet Parasitol Reg Stud Reports*. 2017;9:6-8.
13. Neves LB, Teixeira PEF, Silva S, et al. First molecular identification of *Echinococcus vogeli* and *Echinococcus granulosus* (*sensu stricto*) G1 revealed in feces of domestic dogs (*Canis familiaris*) from Acre, Brazil. *Parasit Vectors*. 2017;10:28.
14. Kuru B.B., Aypak S. and Aysul N. Prevalence of *Echinococcus granulosus* determined with Polymerase Chain Reaction in Dogs in Aydın District. *Turkiye Parazit Derg* 2013;37:78-83.
15. Mulingea E., Magambob J., Odongoc D. et al. Molecular characterization of *Echinococcus* species in dogs from four regions of Kenya. *Vet Parasitol*. 2018;255:49–57.
16. Grech-Angelini S, Richomme C, Peytavin de Garam C, et al. Identification and molecular characterization of *Echinococcus canadensis* G6/7 in dogs from Corsica, France. *Parasitol Research*. 2019;118:1313–9.
17. Keyhani A, Sharifi I, Bamorovat M, et al. Epidemiological and molecular studies on *Echinococcus granulosus* from free-roaming dogs in Southeast Iran. *Vet World*. 2020;13:739-45.
18. Sharma PM, Thapa NK, Tshomo P. Occurrence of *Echinococcus granulosus sensu lato* and Other Taeniids in Bhutan. *Pathogens*. 2021;10:330.
19. Awosanya EJ, Ligali Z, Duedu KO. Prevalence of *Echinococcus granulosus sensu lato* in Owned Dogs in Lagos State, Nigeria. *Vet Sci*. 2021;8:101.