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# Preliminary studies on the prevalence and genotyping of *Echinococcus granulosus* infection in stray dogs in Van Province, Turkey

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## Abstract

**Introduction:** *Echinococcus granulosus* is a zoonotic helminth of the *Taeniidae* family living in the small intestines of dogs. The hydatid cyst, which is the larval form of this parasite, is observed in sheep, goat, cattle, and many other organisms including humans. It causes a disease called cystic echinococcosis. Identification of strains of *E. granulosus* in dogs is critical in parasite control and eradication where possible. This study aims to determine the genotype of *E. granulosus* eggs and prevalence of this parasite in the faeces of dogs in the Van Province using the copro-PCR method. **Material and Methods:** This study was conducted between 2015 and 2016 on the faeces obtained from 100 stray dogs from different parts of the Van Province. The coprological examination was conducted using the formalin-ether concentration method. **Results:** *Taeniidae* eggs were found in 10 (10%) out of 100 faecal samples. *E. granulosus* was detected in 4 out of 10 of these (40%) infected samples. Sequence analysis of positive amplicons obtained from PCR showed that there were sheep strains (G1). **Conclusion:** Dogs in Van area are primarily infected with the livestock genotype of *E. granulosus*, which is thought to be a potential zoonotic threat to humans.

**Keywords:** dogs, *Echinococcus granulosus*, PCR, genotyping, Turkey.

## Introduction

Echinococcosis is the general name which expresses the entire pathology of the disease caused by both adult *Echinococcus* spp. in the definitive host (canids and felids) and the larval form in the intermediate hosts (ungulates and humans). However, *Echinococcus* spp., which are carnivore parasites, originate from metacestode forms in herbivore animals and humans as the intermediate host, where the disease is termed "larval echinococcosis". This zoonotic disease is a serious threat to public health. Dogs become infected by eating organs such as the liver and lungs infected with fertile cysts. Humans are affected by parasitic eggs due to a contaminated environment or close contact with infected dogs. The prevalence of the disease in developed countries has been reduced in recent years with the development of public education, improved hygiene conditions, regular anthelmintic drug applications, and the wide use of ready-made dog food. However, hydatidosis remains a critical parasitic disease and a socio-economic problem for humans and animals in many parts of the world, except countries and regions where control programmes have been successfully introduced (3, 32).

Hydatidosis is a medical problem in Asia, the Mediterranean countries, South America, Africa, and China; and the prevalence of the disease has increased in urban areas within the European Union and varied in different regions in recent years (17). It has been noted that the infection may be detected sporadically outside the endemic regions, but the parasites are not observed in Greenland or Iceland (18). Cases of human cystic echinococcosis are widely distributed in Turkey, being reported from health institutions and organisations throughout the country. The revealed prevalence of cystic echinococcosis (CE) in Turkish livestock ranges from 3% to 46.4% and varies in different regions (14, 23, 24, 29). On the other hand, the prevalence of the disease has been reported as 0.94% to 54.5% in research conducted in different regions of Turkey on the presence of *E. granulosus* in dogs (4, 16, 25). *E. granulosus* occurs as several distinct genotypes (designated G1–G10) infecting different domestic

and/or wildlife intermediate host species including humans (6, 32). In Turkey, the common sheep strain G1 of *E. granulosus* is known to occur in humans and livestock other than sheep such as goats and cattle. Identification of different strains in the genus *Echinococcus* is of great importance for epidemiological understanding and control of hydatid disease (15, 27, 30, 31). The total cattle, sheep, and goat populations in Van Province in Turkey are 179,223, 2,670,576, and 275,277 animals, respectively (28).

Dogs are at the top of the list of factors that should be considered in control of the disease, as they are in close daily contact with domestic ruminants and humans. Dog faeces are a heavy determinant in the spread of *E. granulosus* eggs in the environment. Therefore, detection of the prevalence of echinococcosis in dogs is of prime importance in operating an effective control programme (7).

Eggs of *E. granulosus* detected by routine microscopic techniques cannot be distinguished from *E. multilocularis* or *Taenia* spp. Several methods have been applied for the accurate diagnosis of *E. granulosus*-infected dogs. Among these methods, copro-PCR for distinguishing species of taeniid eggs in faecal or environmental samples has begun to be used commonly (1, 3, 9, 20, 21, 23). Although several PCR studies have indicated the prevalence of canine echinococcosis in Turkey (12, 16, 25, 33, 34), PCR-based molecular analysis of canine echinococcosis is still rather limited (2, 19, 23). To the best of our knowledge, there is no report in the literature demonstrating the prevalence of canine echinococcosis in Van Province. The aim of this study was to determine *E. granulosus* egg dissemination and to genotype *Echinococcus granulosus* isolated from stray dogs using copro-PCR in Van Province, Turkey.

## Material and Methods

**Collection of faecal samples.** This study was conducted between 2015 and 2016 on faeces obtained from a total of 100 stray dogs from different parts of Van Province. Samples were randomly collected in public areas (preferably in areas attended by people, like parks and other public places) and from dogs of different breeds and ages (not registered). The obtained faeces were brought to the laboratory on the same day and kept at −80ºC to keep the eggs inactive until they were used (18). Macroscopic examination of the faeces was carried out by observation of the visible outer surface under sufficient light and by inspecting the inner surface by crumbling the faeces with a glass rod to ascertain existence of cestodes. The formalin-ether concentration method was applied (22), then the presence of taeniid (*Taenia* spp., *Echinococcus* spp.) eggs was assessed using an inverted microscope. All these samples, including taeniid eggs, were selected for further molecular studies.

**Molecular analysis.** DNA was extracted from microscopically positive specimens in PCR analysis. Due to the significant amount of protein and nucleic acid contained in the faecal sample, it was thought that the addition of lysis solution, proteinase K (20 mg/mL, Sigma-Aldrich, USA) and 10% sodium dodecyl sulphate (SDS) to the original ZR Fecal DNA miniprep kit would increase the sensitivity. Accordingly, faecal samples were dissolved in this solution at room temperature (20–25ºC). The precipitate in the Falcon tubes was vortexed by adding distilled water in a volume of 200 µL to the 2 mL ZR Bashing Bead (Zymo Research, USA) lysis tubes (with 0.1 and 0.5 mm beads) contained in the kit, which were filled with 100 µL of vortexed faecal sample and 280 µL of lysis solution and the contents were vortexed again for 20 min. Subsequently, 80 µL of proteinase K and 40 µL of SDS were added to the tubes and incubated in a water bath for 24 h at 56°C. Then the samples were centrifuged at 13,000 rpm for 10 min. The supernatant obtained at the end of the centrifugation was used for DNA extraction. The extraction procedure was then continued. Final elution of DNA was adjusted to 50 µL. The obtained extracts were stored at −20ºC until studied in PCR. Genomic DNA extracts from faecal samples were subjected to JB3-(5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5-(5′-TAAAGAAAGAACATAATGAAAATG-3′) specific primer PCR reactions amplifying the gene region of 446 bp of *E. granulosus* cox1. PCR was carried out in a final volume of 25 µL (6), containing: 7.5 µL DNase- and RNase-free sterile distilled water (Biobasic, USA), 10 µL 5× MyTaq Reaction buffer, 1 µL of each primer (20 pmol), 5 µL of template DNA (100–200 ng), and 0.5 µL of TaqDNA polymerase (1.25 IU) (MBI Fermentas, Canada). The PCR conditions were as follow: 5 min at 94°C (initial denaturation), 35 cycles of 30 s at 94°C, 45 s at 50°C, 35 s at 72°C, and finally 10 min at 72°C (final extension). The PCR products were separated on agarose gels (1.5%), stained with ethidium bromide and visualised and photographed on a UV transilluminator.

In PCR, DNA previously identified as originated from a sheep strain (accession number MF544127) by DNA sequence analysis was used as a positive control and distilled water as a negative control. PCR products encoding the cox1 (mt-DNA) gene region and primers were packaged neatly for sequence analysis and sent to the Sentegen company in Ankara. The purification and gene study of the products were performed by this company. Alignment was performed for the cox1 (mt-DNA) gene region, and sequence files obtained were processed with BioEdit software (Tom Hall of Ibis Therapeutics, USA). The data received from GenBank for the cox1 (mt-DNA) gene region was added and the alignment was performed using the ClustalW module within the BioEdit software. The resulting aligned basis sequences were processed using the MEGA 7 package to give the calculation of

maximum probability analyses, Jukes-Cantor model genetic distance parameter, and bootstrapping analysis through 1,000 replicates. Phylogenetic trees were constructed with the neighbour joining (NJ) algorithm using the specified models, and their topologies were compared. The specimen *Taenia multiceps* (AB792725) was used as an external group.

## Results

Out of 100 samples examined, *Taenia*-type eggs were found in 10 (10%) of faecal samples (Fig. 1). Some helminthic and protozoal parasites such as *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis*, *Ancylostoma* spp., *Isospora* spp., and *Sarcocystis* spp. were also found.

Of the 10 taeniid isolates identified by PCR with the use of species-specific primer pair, four (40 %) samples were shown to be *E. granulosus* (Fig. 2).

A sample obtained from a dog in the Province was confirmed to be infected with *E. granulosus*. The sequence was deposited into GenBank under accession number MF544126. The nucleotide alignment of sequences was analysed in comparison with *Echinococcus* strains deposited in GenBank (Fig. 3).

Comparison showed that the sample sequence was identical to the domestic sheep strain G1 (DQ062857). Guanine (36) was replaced by thymine in G2, while no variation was detected in G1. Two variations were found in the *E. granulosus* cox1 region of stray dogs when compared with G3 (JX878692). Phylogenetic analysis revealed a robust tree associating our isolate of G1 genotype with the same sister group as a variety of G1 genotype (common sheep strain) sequences from different geographical regions of the world, although it was more genetically related to the Portugal isolate (Fig. 4).

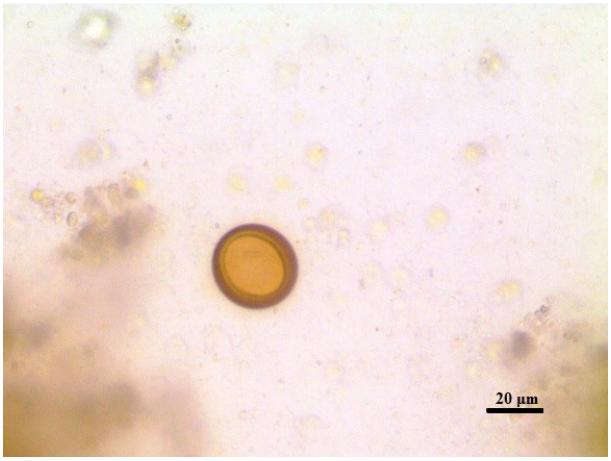


**Fig. 1.** Taeniid eggs detected in dog faecal samples

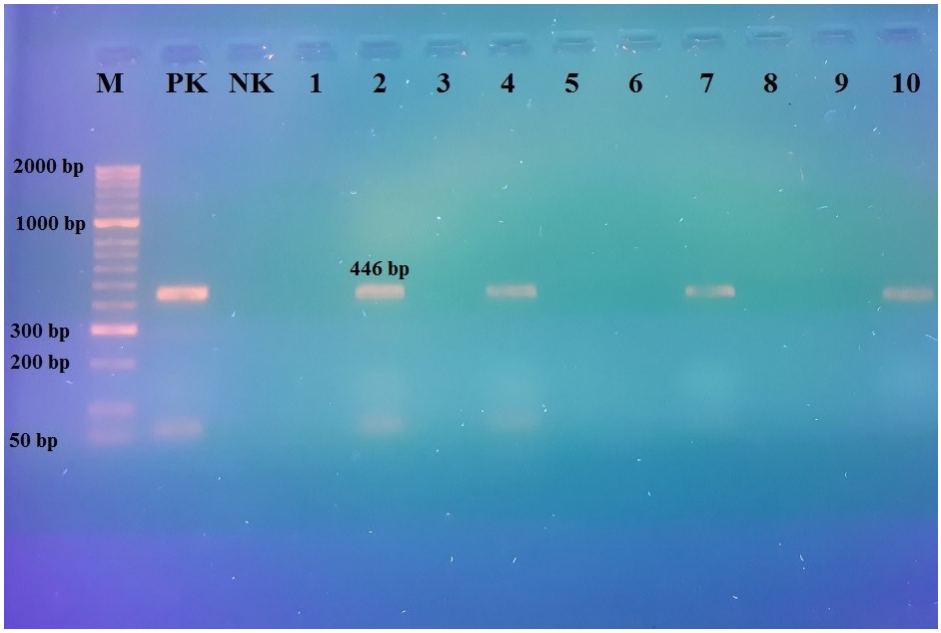


**Fig. 2.** Gel electrophoresis from PCR amplification of *E. granulosus*. M – molecular marker 50 bp DNA ladder (HyperLadder), NK – negative control (no DNA), PK – PCR products from *E. granulosus* protoscolex (positive control), lanes 2, 4, 7, 10 – specific product for *E. granulosus* isolated from dog faeces

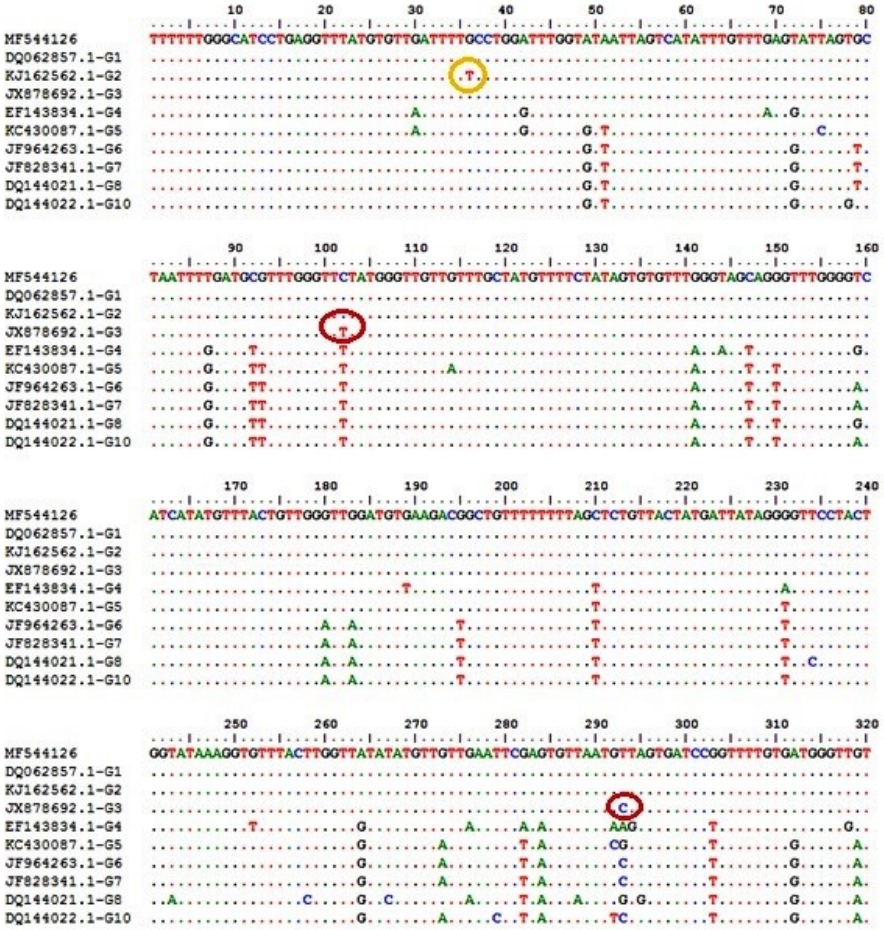


**Fig. 3.** Multiple sequence alignments of *E. granulosus*. Cox1 gene based on *Echinococcus* strains available from GenBank. Dots indicate nucleotides that are conserved relative to the published sequence

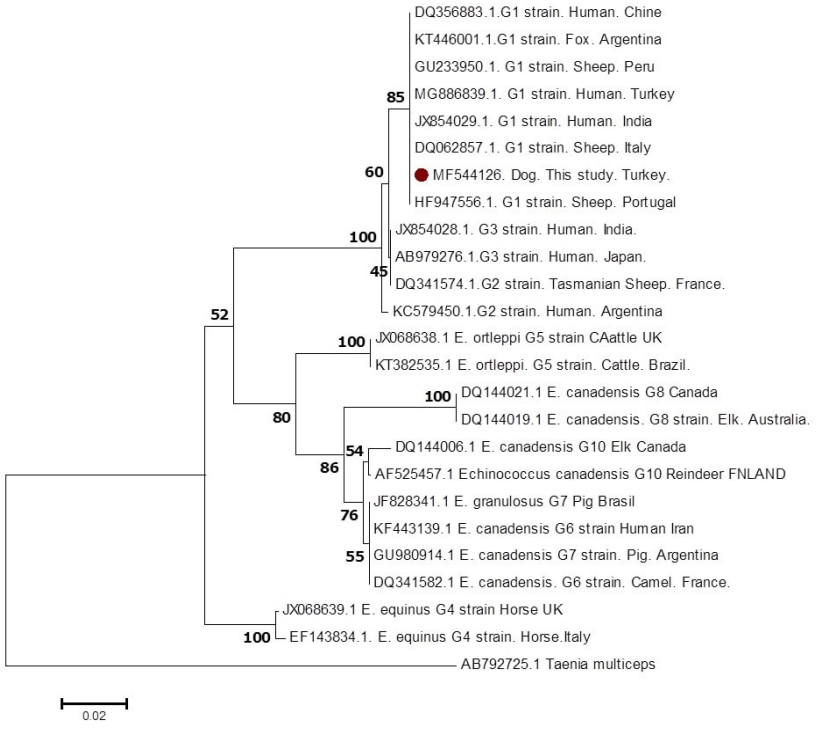


**Fig. 4.** Phylogenetic tree sequences of G1 and G10 genotypes of *Echinococcus* species and their relationships with the reference sequences (red colour) of both genotypes from GenBank. The tree was obtained from partial sequencing of Cox1 gene. The scale bar indicates the proportion of sites changing along each branch

## Discussion

*Echinococcus granulosus*, *E. multilocularis*, *E. oligarthrus*, and *E. vogeli* are the four commonly accepted species within the *Echinococcus* genus. However, two new species have been identified in recent years. These are *E. schiquicus* and *E. felidis* found in foxes on the Tibetan plateau and in lions in Africa, respectively. *E. granulosus* and *E. multilocularis* are more common than the others and are known to be the most crucial disease factors in humans and animals. These are also the species found in Turkey (1, 26). Cystic echinococcosis or hydatidosis is a neglected cyclozoonotic disease affecting humans and their livestock, causing significant socioeconomic and public health detriment, mostly in developing countries (35). Human hydatidosis cases are high in number in Turkey, being recorded from health institutions and organisations throughout the country; however, very few efforts have been put into the research and control of echinococcosis (31). Determining the infection rate in dogs is significant for epidemiologic studies and surveillance of control programmes. The traditional standard method used for surveys of *E. granulosus* infection in dog populations is arecoline purging. Although the specificity of the method is quite high, its sensitivity rate has been reported as 78% (26). In more recent times, a copro-ELISA test for the parasite antigens in faeces has been developed for diagnosis. There are several advantages such as specificity of approximately 97% to coproantigen-based detection of *Echinococcus*. However, sensitivity is relatively limited. Much research has shown that this method may not be sensitive enough for detecting coproantigens in faecal samples of animals that contain a low number of tapeworms (8, 10, 11, 13).

The diagnostic value of faecal examination is very low, and the eggs of *Echinococcus* and *Taenia* species cannot be distinguished morphologically by conventional flotation techniques. The copro-DNA method is a precise and sensitive method for the detection of *Echinococcus* infections in live animals and is based on the principle of detection of DNA originating from the ring, eggs, and cells of the parasite. In areas where *E. granulosus* and *E. multilocularis* are co-endemic, this method can positively distinguish the species (26). PCR using the U1 snRNA gene revealed that even one egg in 4 g of faeces in *E. multilocularis* infections could be detected, and the sensitivity of the method is 100%. In recent years, a copro-PCR has been developed for the detection of *E. granulosus* using the Mt-cox1 and EgG1 Hae III genes. It has been reported that there is no cross-amplification with *Taenia hydatigena*, *T. ovis*, *Dipylidium caninum*, or *E. multilocularis* (1). In previous studies from around the world, the prevalence of *E. granulosus* was found to be between 3.6% and 25.9% by molecular biological techniques (21). The present study using the copro-PCR technique revealed that *E. granulosus* infection is present in 4% of dog faecal samples in Van Province. Although extensive studies have been carried out in the world on the determination of the species of canine echinococcosis at the molecular level (3, 9, 21, 22), few researchers have used the copro-PCR test in Turkey.

In the last few years, the identification of *E. granulosus* eggs has been performed in molecular biological studies (2, 19, 23). In Mus Province, it was revealed using PCR for the first time that 9% of dogs were infected with *E. granulosus* (2). In a recent study in Aydin, 1% of household dogs were found to be infected with this cestode (20). As a result of a PCR performed with specific primers that replicated the cox1 gene region, Oge *et al*. (23) reported that 14% of dogs were infected in the province of Ankara. In the present study, four dogs (4%) were infected with *E. granulosus* as demonstrated by PCR. The low prevalence of *E. granulosus* in the present study may be related to the weaker presence of favourable factors like temperature, environmental conditions, improper disposal of infected organs and dead animals, practices like backyard slaughtering, and number or movement of stray dogs. In addition, this study indicated that not only was *E. granulosus* in dogs, but the dogs with low as well as high levels of infection with this parasite can be the source of the spread of the infection to its intermediate hosts, *i.e*. livestock and humans.

The present study is the first genetic characterisation of *Echinococcus* isolates from canine faecal samples in Van Province, Turkey. Molecular analysis shows that *E. granulosus* found in stray dogs in the province belonged to genotype G1 (sheep strain) and was 100% homologous to *E. granulosus* genotype 1 sequence DQ062857. It appears that the predominant genotype is G1 in the Van area, and further sampling of livestock animals is required for more extensive investigation of transmission of *E. granulosus* G1. Identification of the dominant strains in the endemic regions and determination of the prepatent periods of these strains is also vital for ensuring efficacy in control programmes with the use of anti-parasitic drugs (30).

As a conclusion, the contamination of dog faeces in Van Province with *E. granulosus* is demonstrated at the molecular level for the first time. These faeces also pose a potential risk to human health. For this reason there is a need for centrally coordinated control and prevention methods against the infection mentioned in this study.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

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