T.C
FIRAT UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF VETERINARY PARSITOLOGY

MOLECULAR CHARACTERIZATION OF CATTLE AND SHEEP HYDATID CYST SAMPLES OBTAINED FROM SLAUGHTERHOUSES IN ELAZIĞ

MASTER'S THESIS

SARFRAZ MEHMOOD

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ETHICAL STATEMENT

I have carried out this thesis with my own studies, from the planning of the studies to the acquisition of findings and writing, I do not have any unethical behavior in all stages, I hereby declare that I have obtained all the information and data in this thesis within the academic and ethical rules, and that I refer to the data, information and interpretations included in this thesis but which are not among the findings of this thesis.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

CE	Cystic Echinococcosis
AE	Alveolar Echinococcosis
PE	Polycystic Echinococcosis
MRI	Magnetic Resonance Imaging
ELISA	Enzyme Linked Immunosorbent Assay
CO1	Cytochrome c Oxidase 1
RAPD	Random Amplification of Polymorphic DNA
PCR-SSCP	PCR-Single Strand Conformation Polymorphism
bp	Base Pair
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Mgcl2	Magnesium Chloride
dNTP	Deoxyribonucleotide Triphosphate
WHO	World Health Organization
FFPE	Formalin Fixed Paraffin Embedded

1. ABSTRACT

Echinococcosis is a life threatening zoonotic infection, recognized as seventeen neglected tropical diseases in the world by WHO, caused by the larval stage of cestodes species of genus *Echinococcus* and is responsible for significant economic as well as medical losses each year. Different molecular techniques have been used to study the genetic variations of *Echinococcus* spp, investigations have been made using different nuclear as well as mitochondrial regions for genotyping of the *Echioncoccus* spp. The purpose of this study is the identification, molecular analysis and genotyping of the *Echinococcus* spp. in sheep and cattle samples collected from slaughterhouse of Elazig, Turkey by using 446 bp of mitochondrial cytochrome c oxidase subunit 1 (mt-CO1) gene. 90 samples have been selected on the basis of cyst quality and PCR band quality and the samples were then sent for gene sequencing. 5 samples were not sequenced properly so alignment has been performed on 85 samples (19 sheep, 66 cattle) to identify the species of isolates. Out of 85 isolates, 84 were recognized as *E. granulosus sensu stricto* and one sheep isolate was found to be G6 genotype of *E. canadensis* which is identified for the first time in Turkey. However single nucleotide polymorphism has been observed in not only samples of different animals but also in samples collected from same cattle. Multiple organ infection has been observed in two of the cattle both in lungs and liver. As a result of haplotype analysis, 16 haplotypes of *E. granulosus s.s.* have been detected in 66 cattle isolates whereas 7 haplotypes of *E. granulosus s.s.* were identified in 18 sheep samples.

Keywords: Echinococcus granulosus, Hydatid cyst, sheep, cattle, PCR, sequence, Elazig.

2. ÖZET

ELAZIĞ'DA MEZBAHANELERDEN TOPLANAN SIĞIR VE KOYUN HİDATİK KİST ÖRNEKLERİNİN MOLEKÜLER KAREKTERİZASYONU

Echinococcosis, Dünya Sağlık Örgütü tarafından dünyadaki on yedi ihmal edilmiş tropikal hastalıktan biri olarak kabul edilen, *Echinococcus* cinsinin cestod türlerinin neden olduğu ve her yıl önemli ekonomik ve tıbbi kayıplardan sorumlu, hayatı tehdit eden zoonotik bir enfeksiyondur. *Echinococcus* spp'nin genetik varyasyonlarını incelemek için farklı moleküler teknikler kullanılmış, *Echinococcus* spp'nin genotiplenmesi için farklı nükleer ve mitokondriyal bölgeler kullanılarak araştırmalar yapılmıştır. Bu çalışmanın amacı, *Echinococcus* spp'nin tanımlanması, moleküler analizi ve genotiplenmesidir. Elazığ'daki mezbahalardan toplanan koyun ve sığır örneklerinde 446 bp mitokondriyal sitokrom c oksidaz alt birim 1 (mt-CO1) geni çoğaltılmıştır. Neticede PZR band kalitesine göre 90 örnek seçildi ve daha sonra örnekler gen dizilimi için ticari firmaya gönderildi. Bu çalışmada 5 örneğin sekansı iki kez yapılmasına rağmen başarılı olunmadı, bu nedenle izolat tanımlamak için 85 numuneye (19 koyun, 66 sığır) sekanslama yapıldı. Sekans analizi sonucunda, 85 izolattan 84'ü *E. granulosus sensu stricto* olarak belirlenirken bir koyun izolatının, Türkiye'de ilk kez bildirilen *E. canadensis'*in G6 genotipi olduğu tespit edildi. Bununla birlikte, sadece farklı hayvan örneklerinde değil, aynı sığırlardan toplanan örneklerde de tek nükleotid polimorfizmi gözlemlendi. Sığırların ikisinde hem akciğerde hem de karaciğerde çoklu organ enfeksiyonu gözlendi. Haplotip analalizi neticesinde ise, 66 sığır izolatında *E. granulosus s.s.*'un16 haplotipi tespit edilmiş, 19 koyun örneğinde 7 haplotip belirlenmiştir.

Anahtar Kelimeler: *Echinococcus granulosus*, Kist hidatik, koyun, sığır, PZR, sekans, Elazığ.

3. INTRODUCTION

3.1. Cystic Echinococcosis

Echinococcosis is a life threatening zoonotic disease, caused by the larval stage of cestodes species of genus Echinococcus. Studies have showed the three different forms of disease in humans i.e., cystic echinococcosis (CE), alveolar echinococcosis (AE) and polycystic echinococcosis while the two of them CE and AE are the most important medical forms (1; 2). Initially *E. granulosus* was considered only the causative agent of CE, later there were nine different species recognized through recent advances of phylogenetic systematics having differences in adult worm morphology, specificity of host, and pathogenicity: *E. granulosus sensu stricto* (G1-G3), *Ecinococcus equinus* (G4), *Ecinococcus ortleppi* (G5), *Ecinococcus canadensis* (G6-G10). Besides *E. felidis, E. multilocularis, E. vogeli, E. oligarthrus* and *E. shiquicus* are the other species in Echinococcus spp. (3; 4). Different species of *Echinococcus* spp. cause different diseases in the intermediate hosts, *E. granulosus s.s., E. equinus, E. ortleppi,* and *E. canadensis* causes CE, while *E. multilocularis* causes AE and *E. vogeli* and *E. oligarthrus* causes polycystic echinococcosis (5; 6). The prevalence of the CE is about 1/100.000 in developed countries, studies have showed approximately 2-3 million of human cases affected by CE worldwide (7). However it was reported worldwide that (88.44%) of human CE cases are caused by *E. granulosus s.s.* (G1-G3), while G6 and G7 of *E. canadensis* causes 7.34% and 3.73% of human infections respectively (8). The taxonomy of the genus *Echinococcus* was reviewed in order solve the systematic status of various species and genotypes (5; 9).

However, the conflict of phylogenetic relations within *E. canadensis* (G6-G10) are still debatable. Studies have suggested the division of *E. canadensis* into two sub-species *E. canadensis* (G8/G10) and *E. intermedius* (G6/G7) (10; 11). *Echinococcus granulosus s.s.* and *E. canadensis* are the two more prevalent species responsible for CE in human worldwide (8; 12).

3.2. Morphology of the worm

Echinococcus spp. shows much resemblance in morphology and development. The adult worms shows variations in morphology as the smallest adult tapeworms is of less than 2 mm whereas largest worm is 1 cm having a scolex, neck and 3-5 proglottids, the anterior proglottid are immature, the middle proglottids are mature while the posterior proglottid contain fully developed eggs and is gravid (5). The shape of the rostellar hooks, genital pore's location, the arrangements of hermaphroditic reproductive organs and the number of proglottids vary within species (13).

3.3. Life cycle of *Echinococcus granulosus*

Cystic Echinococcosis is transmitted between two hosts, the intermediate hosts including herbivorous and omnivorous mammals and the definitive host usually dogs or other canids (14). Pathogen affects liver the most forming hydatid cysts in approximately 70% of cases, while the second most common location is lungs, however hydatid cysts can also present in other organs of intermediate hosts (15). The larval stage of *E. granulosus* resides inside various organs of herbivore animals like sheep, cattle, goats and camels for long-term called as metacestode stage referred as fluid filled hydatid cysts in internal organs (mainly liver and lungs) and these organisms serve as

intermediate hosts for the parasite (16; 17). Humans are considered as accidental hosts and once humans ingest food or water contaminated with infective eggs of *E. granulosus*, they will develop the disease and will serve as intermediate hosts for parasite (18). Adult *E. granulosus* resides in small intestine of dog which is the definitive host of the parasite, once offal containing mature metacestode stage (hydatid cysts) eaten, which may contain protoscolices and each of them has the potential to develop into adult worm inside dog's intestine. The matured worms then lay eggs which are released out to environment via dog's feces. Humans and other herbivore animals get infected by eating vegetation and drinking water contaminated by infective eggs (19; 20; 21). Thousands of eggs are released by the gravid proglottid segment daily which then released out through feces of the animal and an intermediate hosts (usually sheep, cattle, goat, buffalo etc.) or accidental host (humans) subsequently ingest them. The eggs hatch to release oncosphere once reached in intestine of intermediate or accidental host and then penetrate into circulation through the intestinal mucosa. Once reached in the liver or other organs, they develop into hydatid cysts and starts to grow and gets enlarged and produce protoscolices. Once the infected organs containing cysts eaten by definitive hosts (dogs, canids), the protoscolices attach to the intestinal mucosa where they develop into adult worms to complete life cycles (20; 22; 23). A complete life cycle takes about 4-7 weeks. As for transmission of the disease both the definitive and intermediate hosts are required as eggs to hatch and cysts to develop. For this reason, human-to-human transmission is not possible (23).

The hydatid cyst which is spherical sac filled with fluid containing protoscolices consist of three layers: inner most layer of parasite cells called as germinal layer, the acellular laminated membrane of variable thickness and an outer most layer which is called as host layer as the layer is produced by host itself by granulomatous adventitial reaction. Protoscolices are produced asexually by brood capsules (small vesicles bud internally from germinal layer), each protoscolex has the ability to become adult worm if ingested by definitive host (20). It was reported in recent studies that the hydatid cyst develops at slow rate approximately 1-5 mm in diameter per year and usually infections might be acquired in childhood and symptoms appear in adulthood in most cases and thus diagnosed at that time. Mostly single cyst infections in humans have been reported (20; 24), however studies have reported the multiple cysts about 20-40% which may also involve multiple organs (16; 24). The slowly growing hydatid cysts may attain several liters of volume of hydatid fluid containing hundreds of protoscolices in humans as well as in herbivores (20).

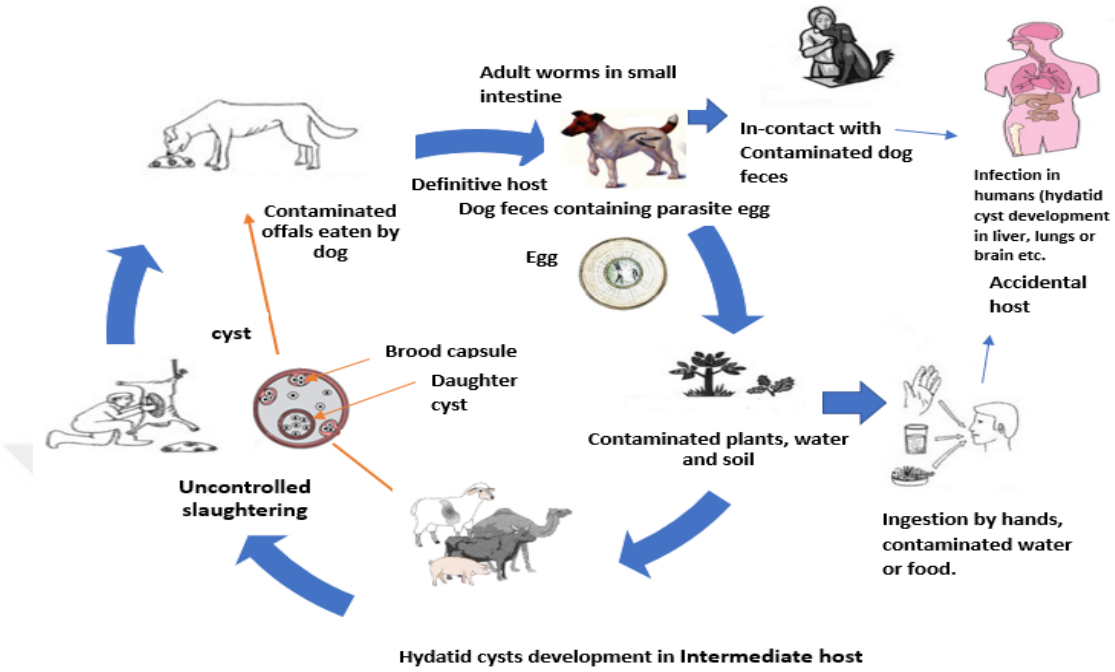


Figure 1. Life cycle of Echinococcus granulosus (Original)

3.4. Distribution

Echinococcus granulosus has impact on livestock as well as on humans worldwide and found in all continents, with highest prevalence especially in the Russian Federation and adjacent independent states, China, Australia, North and East Africa and South America (25). The emergence of the disease in humans is quite evident Central Asia, Eastern Europe, China and Israel (26; 27). Studies showed the high rate of infection in population involved in sheep farming which shows the dog-sheep cycle and the transmission of sheep strain to people and their relevance with the public health (28; 29). However, sylvatic cycles of wild animals as well as the lifecycle patterns of ungulates and domestic dogs have their own zoonotic importance (29). In Alaska two

severe human cases have been reported with infections (in liver) by *E. granulosus* (29; 30).

3.5. Clinical symptoms

The small cysts don't induce major disease and remains in initial phase asymptomatic for many years. The incubation period of the disease continues for month to years however this remains unclear (31). On rupturing or exerting a mass effect by the cysts the infection become symptomatic. According to the study *E.granulosus* affects liver mostly as compared to lungs or hydatiosis occurs both in some cases, however the chances of hydatiosis in other organs are very low about 2-3% in skin, muscles brain, heart, kidney, spleen and rarely in ovaries (1% or less) (32). The severity of the diseases is not only based on the location but also the size of the cysts and their position within organ also plays role in severity. On rupturing of cysts and the cysts leakage, systemic immunological responses have been observed.

3.6.Diagnosis

If CE diagnosed early, treatment of the disease can be initiated early which results chances of cure. As mentioned above, early stages are mostly asymptomatic, so cheap diagnostic methods needed to be use at large-scale screening of population that are at high risk. There are some physical imaging methods for the definitive diagnosis for most cases of CE in man, such as radiology, ultrasonography, computed axial tomography (CT scanning), and magnetic resonance imaging (MRI), The criteria of radiology intended to standardize reporting for clinical trials (13). Immunodiagnosis which is very useful method for the primary diagnosis as well as for the follow-up of

patients for those who undergoes surgeries or pharmacological treatment (13; 31; 33-36). Antibody detection in infected serum shows more sensitivity as compared to the circulating antigens in serum of *E. granulosus*. Most commonly used immunological methods are ELISA, indirect haemaglutination antibody assay, latex agglutination test, arch-5 immunoflurescence, immunofluorescence antibody test and immunoblot test. (37). For immunodiagnosis of CE the hydatid cysts fluid antigens are much useful source (38). There are limited researches made for the development of immunodiagnostic techniques for *E. granulosus* in domestic animals as compared to humans. Diagnoses of CE in intermediate hosts is based on necropsies. Moreover antibody response against infection in natural intermediate is weak as compared to human antibody response against infection (34). Antibodies against antigens including antigen-5 is detectable in the serum of some of infected sheep not all ("non-responders") (34). Three ELISA-based assays showed great effectiveness for detection in sheep by crude *E. granulosus* protoscolex preparation (39). This test in much useful in detection of infected sheep which were studied in groups, however the test is not reliable for the identification of infection in individual animals. Purgation along with arecoline compounds and necropsy of the small intestine are the two methods which are used extensively in case of dogs. For the foxes and other final definitive hosts necropsy is the method of choice. For the detection of infection in definitive host immunodiagnostic methods such as ELISA-based assays specific for detection on serum antibody and detection of parasite products (coproantigens) in faeces have been developed. (35; 40-43). For the field application, the coproantigen ELISA is useful diagnostic method to

replace necropsies and PCR based tests are much useful for the confirmation of positive coproantigen results and for the diagnosis of animals individualy (40).

3.7.Treatment of CE

The chemotherapy is one of the effective method for CE if diagnosed early, benzimidazole compounds, albendazole and mebendazole, have been used as the keystone for chemotherapy. Through recent studies the disappearance of cyst upto 48% have been observed if treated with albendazole (10 mg/kg in divided doses-usually 400 mg-twice daily) mebendazole (40-50 mg/kg per day in three divided doses) is less effective than albendazole (44). Albendazole sulfoxide which is used as the protoscolicidal metabolite of albendazole, the medicine is given in in three to six 4-week cycles with intervals of 14 days Furthermore the continuous treatment can provide improved efficacy without any adverse effects (45; 46). The non-viability of the cyst increases with duration of the treatment as studies have showed 72% of cysts are non-viable after 1 month and 94% of cysts gets non-viable after 3 months of treatment (47). Using these drugs may cause some side effects, however regular monitoring of leucocytes should be made and liver function tests should also be performed on regular basis praziquantel is being used (25 mg/kg per day) with albendazole for combined treatment of CE. Early trial on man showed improved efficacy over albendazole alone (48). It was observed in recent studies that serum concentration of albendazole sulfoxide increased by four folds because of praziquantel. Upon administration of albendozole in the laboratory animals in their early gestation, teratogenicity has been reported (48).

For the large cysts that have chances of rupture and those present in vital anatomical locations or exerting extensive mass effect, surgery is considered to be the best solution (49).

3.8. Prevention and Control of CE

For the prevention of infections one should take some precautionary measures e.g avoid of contact with dog or feces of fox, washing hands and improvement in sanitation, reducing the number of stray dog or fox populations, treatment of dogs praziquantel or use of praziquantel-impregnated baits, proper disposal of infected organs, improving the personal hygine and organizing awareness programs.

EG95 which is a 16.6 kDa protein extracted from *E. granulosus* that was originally cloned from G1 genotype of *Echinococcus granulosus* (50). Recent studies have showed promising results of this recombinant vaccine against *E.granulosus* isolates and also showed high protection rate in intermediate hosts (51). Studies have shown the effectiveness of vaccine if used independently challenge trials had been undertaken in New Zealand, Australia, Argentina, China (52) and Romania against G1 strain (53). Studies have found similarities in most of the EG95 genes except the insertion of 7 amino acids in some isolates and this result was in consistent with the previous studies on G1, which conclude the fact that EG95 shares conservative genes in different isolates. In vitro studies have shown high levels of EG95 specific antibodies which effectively kills the parasite (51). Vaccination approach is undoubtedly to be the effective way of controlling CE and the development of a recombinant vaccine such as EG95 against *E. granulosus* in sheep has shown high degree of protection against different geographical

isolates of *E. granulosus* and therefore it also has the potential to prevent the disease in humans as well (54). Similar type of attempts needed to vaccinate definitive hosts. However many experiments to induce immunity through vaccination in dogs has been made with some encouraging results (31)

3.9. Genotypes of *Echinococcus granulosus sensu lato*

3.9.1.Early period

Echinococcus infections in livestock and humans has been known for many years and scientists have given them names in different languages since the distant past, until the modern zoological nomenclature (in 1798) been introduced bringing advances to give order to local names of animals which were not given to them properly. Almost 85 bi- or trinomial latinised names have been reported in published data till 19th century which were based on the morphological appearances of metacestodes and host origin (55). *Hydatigena granulosa* was the first valid name given to the fertile cyst of echinococcus found in sheep in Germany and the name was given by by Batsch in 1786.Genus echinococcus was then established shortly by Rudolphi in 1801 according to which the protoscolices found in fluid filled hydatid cyst were small, round and spiny and thus the *E. granulosus* (combined with its variants) was created. In 1808 Rudolphi explained link of Echinococcus from dog as *Taenia cateniformis* not recognizing the link between metacestode and adult worms. Eventually *E. granulosus,* a common name was referred to all stages of life cycle in the last decade of 19th century, although similar names like *Taenia echinococcus* were continued in use for some time. Previously many names were assigned to cysts on the basis of adult morphology. Even scientists

considering *E. granulosus* and *E. multilocularis* as different species couldn't defend themselves until *E. multilocularis* lifecycle was discovered in 1950s almost at same time on St. Lawrence Island off Alaska and in central Europe (55; 56).

3.9.2. Species and Subspecies

The debate regarding to echinococcosis infection either caused by one or more Echinococcus species was undergoing in the meantime there were other Echinococcus species been described based on morphological appearances of adult parasite i-e morphology of rostellar hook, number of proglottids. Moreover *E. granulosus* and *E. multilocularis* recognized on the basis of metacestoed-*E. oligarthra* was given name as Taenia by Diesing in 1863, later the synonym *E. cruzi* was described on the basis of metacestode (as Taenia) (57). *Echinococcus longimanubrius* and *Ecinococcus minimus* were also being found in African wild dog and Macedonian wolf respectively. (57- 59). *Echinococcus cameroni* worms isolated from a British fox that had been identified as *E. granulosus* earlier and *E. lycantis* (African wild dog), followed by *E. felidis* from African lion (60; 61). Later on *E. sibiricensis* was synomymised as *E. multilocularis* (62; 63), followed by the description of *E. intermedius* and *E. ortleppi* from domestic dogs in Spain and South Africa respectively (64) and *E. patagonicus* from a wild South American canid (*Lycalopex culpaeus*) (64; 65). *Echinococcus oligarthra* and *E. multilocularis* have been recognized as valid species along with *E. granulosus*, while *E. felidis* and *E. patagonicus* were not given any status because of the lack of sufficient data. Furthermore, five more species were described out of which *E. pameanus* and *E. cepanzoi* were synonymized with *E. oligarthra* and *E. granulosus* respectively (66- 68).

E. russicensis was considered as variant of *E. multilocularis* while *E. vogeli* (69) and *E. shiquicus* (4) are now considered as separate species (9).

In addition to species *E. multilocularis* and *E. granulosus* species were further subdivided into many subspecies based on morphological appearances of the worms. In 1957, Vogel included *E. sibiricensis* in *E. multilocularis* as a subspecies and as well as *E. m. kazakhensis* metacestodes isolated from ungulates was included as subspecies (63; 70; 71). In 1965 major taxonomic revision of *E. granulosus* was performed in which 8 subspecies *E. g. canadaensis*, *E. borealis*, *E. equinus*, *E. felidis*, *E. lycaontis*, *E. ortleppi*, *E. africanus* and *E. newzealandensis* were included (72). Although in 1967 Raunch disproved the taxonomic classification on the basis of the differences in morphological characters to host-induced modification found by previous scientists (73).

3.9.3. Genotypes

There were finally four species recognized at the beginning of 1980s *E. granulosus*, *E. multilocularis*, *E. oligarthra* and *E. vogeli* (74). However *E. granulosus* consisted of number of variants within specie based on specificity of host, biochemical parameters, morphological differences in adult worms, developmental stages and also the geographical distribution. However, there were no attempts made for renaming the subspecies of genetic variants and to describe the variants intraspecific system of 'strains' was introduced on the basis of epidemiological significance (29). Initially the fully developed system consisted of eleven strains (sheep, Tasmanian sheep, buffalo, cattle, horse, pig, variant pig (or human-pig), camel, American cervid, Fennoscandian cervid and lion strain) based on non-genetic characters such as geographic distribution,

host spectrum, morphological differences and aspects of development. In early 1990s partial sequencing played major role to define the variants on the basis of genetic makeup, moreover the sequencing mitochondrial genes (CO1 and NAD1) of seven strains of *E. granulosus* were used study to identify the variants (75; 76). Data sequencing of genome corresponded well to characterize the strains and thus the term genotype introduced for naming (G1-G7) which partially replaces the previous strain names. However later the term "genotype" was considered as synonym of "strains".

Moreover three additional taxa: the American cervid strain (G8), a variant pig (or human-pig) strain (G9) and the Fennoscandian cervid strain (G10) were added previously characterized seven genotypes/strains(G1/sheep strain; G2/Tasmanian sheep strain; G3/buffalo strain; G4/horse strain; G5/cattle strain; G6/camel strain; G7/pig strain) (77- 79).

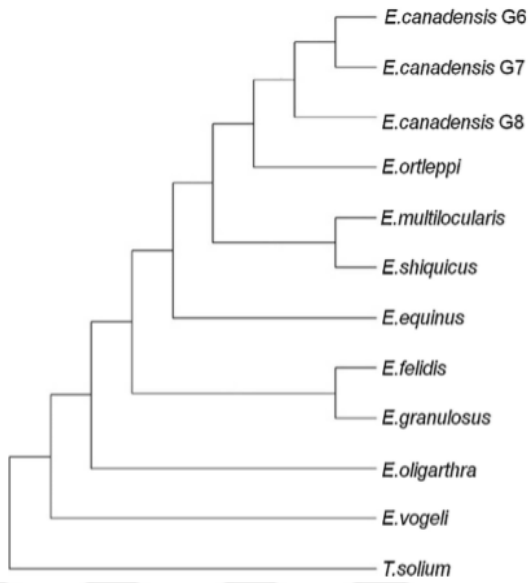


Figure 2. Cladogram of Echinococcus spp. Showing the phylogenetic relationship of the species and was obtained from 5170 nucleotides of the mitochondrial cox1, nad1, rrn and cob gene through Maximum Likelihood analysis (12).

Cladogram shows the similarity and divergence among species and genotypes based on the analysis of four mitochondrial genes (cox1, nad1, rrn and cob). *Echinococcus vogeli* and *E. oligarthra* occupied basal position, whereas *E. canadensis* and *E. ortleppi* shared same clade and *E. canadensis* G6 and G7 showed much similarity between each other as compared to G8. *Echinococcus multilocularis* and *E. shiquicus* shared same clade showing similarity between each other. Whereas *E. felidis* and *E. granulosus* were in same clade showing similarities between each other.

After two decades of data accumulation on epidemiology, geographical data and biochemical data on *E. granulosus sensu lato* and the analysis of nuclear and mitochondrial gene sequences as well as the phylogenetic relationship of species, limitations and contradiction of strains/genotypes system, the taxonomic revision of *E.*

granulosus was reviewed resulting the formulation of some postulates; the phylogenetic relation of *E. granulosus* sensu lato compared with *E. multilocularis* and *E. shiquicus* (which had in the meantime been described from the Tibetan plateau) (4; 80), and the genetic variations were in micro variant range between G1-3 and G6-7 of same taxon while G4 is distantly related to G5. Subdivision of *E. granulosus* into four species (81), *E. granulosus equinus* which was given separate specie rank for the horse strain (genotype G4), *E. ortleppi* (64) was restored for the cattle strain (genotype G5) (82) and the name *E. granulosus* was given to the genotypes G1 to G3 (sheep, Tasmanian sheep and buffalo strains), however for camel, pig, cervid and lion strains the issue of allocating scientific names were left unresolved. After five years of complete mitochondrial genome sequencing *E. granulosus canadensis* was given (83) specie rank including closely related genotypes (G6-G10) (84). Now the ongoing taxonomic reshuffle consists of *E. granulosus* sensu lato which is further divided into *E. granulosus s.s.* (G1-G3), *E. felidis, E. equinus, E. ortleppi, E. canadensis,* along with the species of alveolar and polycystic echinococcosis which consist of *E. multilocularis, E. shiquicus, E. oligarthra* and *E. vogeli* (12).

In order to study the genetic variations among different *Echinococcus* spp, different molecular techniques have been used to investigate the nuclear regions encoding rRNA (85). In addition the genetic analysis using mt-CO1 (75) and NAD1 genes (76), PCR-Single Strand Conformation Polymorphism (PCR-SSCP) of CO1 and NAD1 genes (86) and PCR-RFLP of NAD1 (87), regions within the 12S

rRNA gene (88), ATPase subunit 6 (atp6) (4) and the complete mitochondrial genome (84).

3.9.4. *Echinococcus granulosus sensu stricto*

Initially *E. granulosus s.s.* considered as the 'sheep strain' isolated from sheep. Now *E. granulosus s.s.* considered as sheep strain, Tasmanian sheep and buffalo strain (G1,G2 and G3 respectively) (12). G1-G3 were closely related to each other confirmed by the short gene sequencing as well as the sequencing of longer and/or other genes, (9; 89-93). Recent studies have identified 137 haplotypes out of 304 isolates from Europe, western Asia, southern/eastern Asia, Africa, and South America on the basis of complete sequencing of mitochondrial CO1 gene.

Studies have showed the variations in large number of haplotypes of G1, G2 or G3 by using 366 bp sequence of mt-CO1, however these haplotypes belong to same cluster and the whole cluster has a divergence of >100 bp from *E. felidis* which is considered as its sister taxon. Therefore *E. granulosus s.s.* can be used as a name for this cluster. The subdivision of the taxon must be on the basis biological characteristics and nuclear sequence data. Sheep is the primary host of this taxon however other infections with fertile cysts have also been observed from wide range of other animals including wildlife herbivorous species such as equids (29; 94).

There are frequent infections observed in cattle with this taxon though their contribution seem to be less in transmission because the majority of cysts do not reach fertility (95). Human CE is caused by *E. granulosus s.s.* which is considered to be the

most frequent agent worldwide i.e., 88% of infection in human (96). Although there are exceptions in some countries like Sudan and Egypt where *E. granulosus s.s.* is either less prevalent or absent (97; 98).

3.9.5. *Echinococcus equinus*

In 1963, Echinococcus cysts from horse in Britain considered to be *E. granulosus equinus* a distinctive subspecies (99; 100). The G4 genotype was detected Spain and UK from horses and from a donkey in Ireland on the basis of partial sequencing of the CO1 gene as well as comparing the NAD1 mitochondrial sequences (75; 101) and after complete mitochondrial sequencing *E.equinus* was ranked as independent Specie (82; 102). Equidae (horses, donkeys and zebras) are the hosts of the specie and the high specificity of the specie towards its hosts has been observed (9). Infections of G4 have been found worldwide in horse as in UK, Ireland and some central European countries, also recent evidence suggests the presence of *E. equinus* in some of the Eastern European countries (103). Moreover CE as G4 genotype has been also found in horses from South Africa and New Zealand (99) and in United States (US) and Italy (104). Studies have revealed the presence of G4 strain in mule by sequencing mt-CO1 gene (105). The result is in agreement with the study (75), who found the *E. granulosus* G4 genotype as causative agent of CE in equids by sequencing fragments of mt-CO1 gene of 366 bp (75). Moreover another study has revealed the presence of *E. equinus* in donkey from Turkey as the cysts found in 2 year old female donkey and molecular identification and sequencing of a fragment of CO1 and NAD1 confirmed the presence of *E. equinus* (106).

3.9.6. *Echinococcus ortleppi*

Initially the adult worms obtained from dogs in regions of South Africa were identified as *E. granulosus* (60), later described as new species on the basis of morphological differences and also considered to be *E. granulosus* causing CE in cattle (64; 95). Cattle strain of *E. granulosus* showed the differences as compared to other taxa in number of features basis of morphological difference of adult worms, the fertility rate of cysts in cattle, and short development time in dogs (107). Although scientists thought the frequent in cattle raising regions in South Africa, later it was identified from Switzerland and Germany and characterized on the bases of partial sequence of CO1 from a cattle in Netherlands, and designated it as the G5 genotype (72; 101; 107; 108). Now *E. ortleppi* is considered as separate specie which fits in the same clade as *E. canadensis* (9; 82).

Studies have showed the cattle suitable intermediate hosts for *E. ortleppi*, however fertile cysts were also been observed in other species. Fertile cysts have been observed in different regions of the world as in cattle from central European countries (109), in Sudan (88; 110), Kenya (111), South Africa (112), Brazil (113), Italy (89) and most recently in France (114), also infection in cattle as well as in buffaloes in India (86), goats and sheep in Kenya (111), and pigs in India and Kenya (88; 115). Monkeys from Vietnam have been identified by *E. ortleppi* infections (116), and also a caged deer brought UK from France was found infected (117). Studies have reported the presence of *E. ortleppi* infection from seven human isolates in Brazil, Argentina, India, Mexico, Netherlands and South Africa (118).

3.9.7. Echinococcus canadensis

Echinococcus canadensis includes camel, pig and cervid strains. The adult worms of camel strain has shown morphological differences from those of other strains and pig strain has shown similarities with each other (108; 119). Studies have shown the confirmation of specific camel strain in the Middle East as well as Eastern Africa and in Eastern Europe and Mexico pig strain is more prevalent. Based on molecular sequencing pig, camel and cervid strains were considered to be in single species. Genotypes G6, G7 and G8 were allocated through molecular characterization of Echinococcus to cysts taken from African camels and goats, Polish pigs and North American moose (75; 76). Later on genotype G9 was found in human patient in Poland (79), and G10 for the 'Fennoscandian' cervid strain (78). Genotype G9 is now referred as the microvariant of G7 based on ITS1-RFLP results, however the other four genotypes share clade *E. ortleppi* as a sister taxon (78; 120; 121). On the basis of complete sequencing of mitochondrial genome *E. canadensis* was referred to the genotypes (84). This name was retained as an alternative name for the "northern biotype" of *E. granulosus* (73), and other scientific name which was considered as junior synonym was *E. granulosus borealis* (122). Later on due to the differences of host range between domestic camel and pig strain and sylvatic cervid strains and also the geographic differences the proposal of giving them as distinct species emerged and the name *E. intermedius* had been suggested (123; 124). Based on two reported worms from Spanish dog the original morphological description of *E. intermedius* did not matched to the description of worms belong to camel and pig strains (64; 108; 119).The 'domestic' G6/7 and the 'sylvatic'

G8 and G10 are distinct ecologically and geographically, however the argument had been blurred when G6 was identified in reindeer and wolves in Russia (125). Moreover considering the existing mitochondrial data, the resemblance of G10 with G6/7 was more close than G8 which resulted the split in *E. intermedius* (G6,G7) vs *E. canadensis* (G8,G10) which was impossible taxonomically (5; 121; 126). As result the cluster was divided into three species *E. intermedius* (G6/7), *E. borealis* (G8) and *E. canadensis* (G10) (127). With respect to mitochondrial classification the split of species was reliable and can lead to more stable nomenclature. There were some issues which were unresolved and the main concern was the maintenance of the proposed species and genetic identity in sympatric situations and this situation was crucial in regard to independent evolutionary fates of three linages. For this mentioned issue existing data of mitochondrial sequencing appeared to be inadequate, so the need of large amount of data of nuclear genome and more comprehensive studies on biological as well as on adult morphology (5; 9). The differences at molecular level between G6 and G7 observed to be minor because the divergence value of mitochondrial gene was much low and these strains cannot be given separate specie status (5; 9; 112). However goats were found infected with G6 in the province of Argentina while G7 infected the pig which conclude the fact that G6/7 were relevant variants (128).

3.9.8. Molecular Epidemiology of CE in Livestock Worldwide

Molecular epidemiological data obtained from China, India, Iran and Pakistan to the genetic variations of *E. granulosus* in the regions, examine large amount of molecular data available in Iran and India, while genotyping studies in other well know

endemic regions of Asia like Arabian Peninsula and Central Asia are lacking. There are three species circulating in Asian livestock *E. granulosus* s.s., *E. ortleppi*, and *E. canadensis* although *E. shiquicus* is considered to be circulating in wild, in pikas and red foxes as intermediate and definitive hosts, respectively (4). In Asia *E. granulosus s.s.* is the predominant specie identified in livestock. Whereas, *E. granulosus s.s.* co-circulate *with E. canadensis* and/or *E. ortleppi* in India and Iran. However recently two cases of G6 genotype of *E. canadensis* found in two human patients and one in dog in China which suggest the circulation of this genotype in livestock (129). In Iran and India, studies suggested that *E. granulosus s.s.* to be identified in 25-100% of the sheep and (50-100%) of cysts fertility rate has been found, whereas *E. canadensis* G6-G7 were responsible for the remaining infections (115; 130), which suggest the high prevalence of *E. granulosus s.s.* in Asia. However, no fertility data regarding *E. canadensis* (G6-G7) in sheep infections are currently available relatively low number of infections of these genotypes suggest secondary role of sheep in transmission of *E. canadensis*. Although studies in Iran confirmed the presence of G6 genotype of *E. canadensis* in three human cases, and two human cases in Nepal have also been reported (131; 132). Moreover *E. granulosus s.s.* is the only genotype found to be circulating in goats in Asia having fertility rates ranging from 50% to 79% (130; 133). *Echinococcus granulosus s.s.* G1 is responsible to cause CE in bovine (64-100% infections) with fertility rate 18-75%, suggesting that the cattle to be the potential reservoir for human disease (133; 134). Whereas *E. ortleppi* found to be circulating in India (21 to 33%) with a cyst fertility rate of 100% and *E. canadensis* G6 the only genotype identified in Iran with genotype frequency of 6-36%.

There is substantial data of prevalence of genotypes is present in different countries which provides information of genetic variations and geographical distribution of Echinococcus infection in production animals. In Europe there are four Echinococcus species circulating which includes *E. granulosus s.s., E. canadensis, E. equinus* and *E. ortleppi*. Studies have showed the presence of *E. granulosus s.s.* being the dominant specie in Europe. Moreover, G1 of *E.granulosus s.s.* is the only genotype which has been identified in isolates of intermediate and definitive hosts (89; 135). These studies indicate presence of dog-sheep cycle and its dominant role in transmission of the parasite in Europe. Moreover, infection of *E. quinus* (G4) was identified in horses in in Turkey, Italy and Spain, although studies have revealed that this genotype was previously been present in UK, Belgium, Ireland and Switzerland (136). Recent studies showed the molecularly confirmed *E. equinus* infection in an horse in Germany (137) as well as in Turkey. Studies have shown the presence of *E. ortleppi* in Italy however *E. canadensis* (G6-G7) circulate in Greece, Italy, Spain and Turkey and also in Central and Eastern Europe. However in Slovakia and Lithuania these genotypes are the only strains circulating, and this transmission pattern suggests that the dog-pig cycle is predominant in these geographic areas. Pig strain is causative agent for human infections as the increasing number of reports of human patients have been reported (138; 139). In some countries (Italy, Spain and Greece), Studies have reported the presence of *E. canadensis* (G7) in caprine isolates from Greece and Spain, while the infections in goats were mainly caused by G1 and G3 of *E. granulosus s.s* in Italy. The cyst fertility rate in infected goats by G7 genotype of *E. canadensis* varies from 0-16% (140; 141), while infection of G1 genotype of *E.granulosus s.s.* has sterile hydatid cysts (141).

Echinococcus granulosus s.s. (G1-G3) with largely infected the cattle and buffaloes in Europe (142; 143). Detection of sterile hydatid cysts found in cattle, either the infection is caused by G1-G2 (sheep) or the G3 (buffalo) strains (52; 129;141; 144; 145). Whereas *E. granulosus s.s.* infection in buffaloes produce 13-19% fertile cysts reported in the studies (146; 147). Sporadic cattle infections with *E. ortleppi* in Italy has cyst fertility rate of 100% (144). The most frequent Echinococcus strain infection of the horses in Italy, Spain and Germany is the *E. equinus* with genotype frequency 50-100% with highly cyst fertility rate (104; 141; 148). Although there is no human case known to be infected by *E. equinus* which suggests that it may be apathogenic to humans (82; 149). *Echinococus granulosus s.s.* G1 can also infect horses, although resulting with non-fertile hydatid cysts (104). In Slovakia, Romania, and Lithuania CE in pigs is largely caused by *E. canadensis* G7 genotype and 25-88% of the cyst samples were from Italy, and Spain and Hungary, whereas *E. granulosus s.s.* was identified in Bulgaria, Italy, Hungary and Spain with the gene frequency rate ranging from 11% to 100%. Cyst fertility rates vary from 19% to 100% documented in swine CE infections caused by *E. canadensis* G7 (52; 141; 150; 151),whereas cyst fertility rates associated with *E. granulosus s.s.* range from 0-75%, with an unknown cyst fertility rate (115). Moreover, the molecular epidemiological data on buffaloes in Pakistan, India and Iran showed the similar results as those reported in cattle. Thus, studies have proved the presence of *E. granulosus s.s.* as dominant genotype in bubaline CE infections, with *E. ortleppi* being reported only in 14-15% of the infections in India. Cyst fertility rate ranging from 73-100%, independently of the species causing the infection (115; 133). In Asia camels were found to be infected by *E. granulosus* with 67-95% cyst fertility rate however

genotypes G6/G7 of *E. canadensis* infected camel with genotype frequencies ranging from 12-100% (133; 134). *Echinococcus granulosus s.s.* (G1) and *E. ortleppi* have been found to infect pigs in India and the frequency range of infection was 64-100% and 100% respectively (115).

Echinococcus granulosus and *E. canadensis* both have been reported in Algeria, Libya, Kenya, Ethiopia and Tunisia. *Echinococcus canadensis* is the only specie found in Mauritania and Egypt, however in Sudan *E. ortleppi* co-exist with this specie. Data regarding *E. granulosus* genotypes in livestock of Morocco and Tanzania have not been found yet. However recent studies have shown the presence *E. felidis* in wild carnivores, the genotype has been found in lions and hyenas no reports of infection has been found in farm animals (3; 152).

3.9.9. Molecular Epidemiology of CE in Turkey

Until now studies on *E. granulosus* genotypes in Turkey revealed the predominance of G1 genotype. In 1992 sheep isolate from Turkey was analyzed which indicated the presence of G1 genotype, similar results were obtained in 2004 (75; 153). In 2008 formalin fixed paraffin embedded (FFPE) tissues of 20 patients were been examined from Turkey which showed only G1 strain (154). A PCR-RFLP has been used and the mitochondrial CO1 sequence analyses of sheep, cattle, goat, camel, dog and human isolate showed the presence of only G1 strain in Turkey (155). Another study in the same year conducted on sheep and cattle isolates of *E. granulosus* collected from different regions of Turkey, their mt-CO1 gene sequence analysis showed G1 strain of 107 isolates, including isolates from sheep and cattle, and other five isolates including

two sheep and three cattle, were identified as G3 genotype (92). In 2009, two studies have showed the presence of different genotypes of *E. granulosus* in Turkey, first study analyzed sheep and human isolates by sequencing of four mitochondrial genes, the study showed the presence of G1 and G3 genotypes as well as the G7 genotype (pig strain) in three isolates from sheep and humans, the other study identified G1 genotype in Turkish mouflon (91; 156). In 2010 study was conducted on 220 cattle which showed G1 and G3 genotypes, in same year 46 hydatid cysts were collected from humans, the results showed the presence of G1 genotype (157; 158). The study conducted in 2011, on 70 FFPE tissue samples, 26 samples yielded 354 bp of amplification, were analyzed using 12S rRNA PCR with the *E.g.*ss1for. and *E.g.*ss1rev. primers, these samples were identified as G1/G3 cluster. Four samples from remaining isolates yielded 446 bp product out of them one was identified as G3 and two of the samples were identified as G6 genotype (camel strain) (159). Recent study has been conducted on 120 hydatid samples (60 from Elazig and 60 from Erzincan province of Turkey), results have shown the presence of *E. granulosus sensu stricto* in all isolates (160).

Different molecular techniques have been used to study the genetic variations of *Echinococcus spp*, investigations have been made using different nuclear as well as mitochondrial regions for genotyping of the *Echinococcus spp*. Through various identification and characterization methods for species and strains or genotypes within genera Echinococcus have been defined, though some direct DNA approaches in further characterization of genotypes with Echinococcus and solving particular systematic problems such as divergence within specie like *E. granulosus s.s.* (G1-G3). Current

study is based on mitochondrial gene region “cytochrome c oxidase subunit 1 (CO1)”. Considering the previous studies and the present data about *E. granulosus sensu lato*, the aim of the study is the identification, molecular characterization and genotyping of the Echinococcus spp in sheep and cattle samples collected from slaughterhouses of Elazig, Turkey.

4. MATERIALS AND METHODS

4.1. Sample Collection

Hydatid cysts samples were collected from EL-KAS slaughterhouse in Elazig province of Turkey between February and July, 2019. The samples were collected from lungs and liver of sheep and cattle after slaughtering then transferred to molecular parasitology laboratory of Firat University, Veterinary Faculty. Then the hydatid cysts were dissected and the germinal layers (inner most layer) were collected into eppendorf tubes containing 70% ethanol. Besides the cysts collected from same organs were stored in different tubes in order to check any nucleotide differences in the hydatid cysts in same organs. The tubes were stored at -20° till further used.

4.2. Extraction of genomic DNA from germinal layer

In order to obtain genomic DNA from germinal layer, the layer was crushed by surgical blade or scalpel on a glass slide then the tissue sample was placed in the bottom of 1.5 ml nuclease-free micro centrifuge tube following the washing of the samples by PBS (Phosphate Buffer Saline) for at least five times in order to eliminate traces of ethanol and each time discarded the supernatant of eppendorf tubes. For gDNA isolation, gDNA extraction kit (RTA Genomic DNA Isolation Kit from tissue, Turkey) have been used. First we used 200 µl "DL solution" and 20 µl Proteinase-K from gDNA extraction kit and the samples were first mixed by vortexing and then subjected to overnight water bath for incubation at 56°C. After every 30 minutes the samples were mixed by vortexing in order to get effective results of digestion for 2 to 3 times. The

next day the gDNA was extracted by using the protocol mentioned on gDNA extraction kit. A total of 200 µl "solution B" was added and pulse vortex for 20 sec, then briefly centrifuged and incubated for 15 minutes at 65°C in water bath and mix by vortexing after 3 mins. Then 260 µl ethanol (96-100%) was added and mixed by pulse-vortexing for several times then centrifuged briefly and the mixture was transferred to the column inserted in the collection tubes. Mixture was then centrifuged at 5000 g for 1 minute, the collecting tubes containing flow were replaced by new collection tubes. Then 700 µl of "solution W1" was added, centrifuged at 5000 g for 1 minute and collection tubes were discarded and replaced with new tubes. Then 700 µl of "solution W2" was added and centrifuged at 16000 g for 1 minute, tubes containing flow were discarded and replaced with the new collecting tubes. Again centrifuged at 16000 g for 3 minutes in order to remove every particle of ethanol or wash solution from gDNA. Then the spin columns were transferred to 1.5ml collection tubes and 200 µl of "solution E" (elution solution) preheated 70°C was added and the closed tubes were incubate at room temperature (15-20°C) for 3 minutes, then centrifuged at 5000 g for 1 minute and 16000 g for additional 30 seconds. The elute collected in collection tubes included the gDNA.

4.3. PCR reaction

For the amplification of 446 bp gene region of "Cytochrome C oxidase subunit1" (CO1), JB3 and JB4.5 (forward and reverse, respectively) primers have been used. For the identification of Echinococcus specie and the amplification of the 446 bp region of CO1 gene the PCR was performed using gDNA. Reagents used in the PCR reaction are given in Table 1.

Table 1. PCR reaction mixture components for qualitative PCR

Components	**Master mix**
PCR buffer	5 µl
dNTPs (1.25 µM)	4 µl
$MgCl_2$	5 µl
Primer mix (Forward=1ul, Reverse =1ul) (20 pmol from each)	2 µl
Taq DNA polymerase	0.2 µl
Sterile distilled water	28.8 µl
Total	45 µl

The PCR Master mix was prepared for each PCR reaction in order to reduce pipette error and then transfer to PCR tubes. Master Mix (45 µl) transferred to PCR tubes for each sample and 5 µl of gDNA was used for each sample and then vortexed for 10-20 sec following the tubes were shifted to conventional PCR thermal cycler (SensoQUEST). The denaturing, annealing and extending steps with their respective conditions were shown in figure 3.

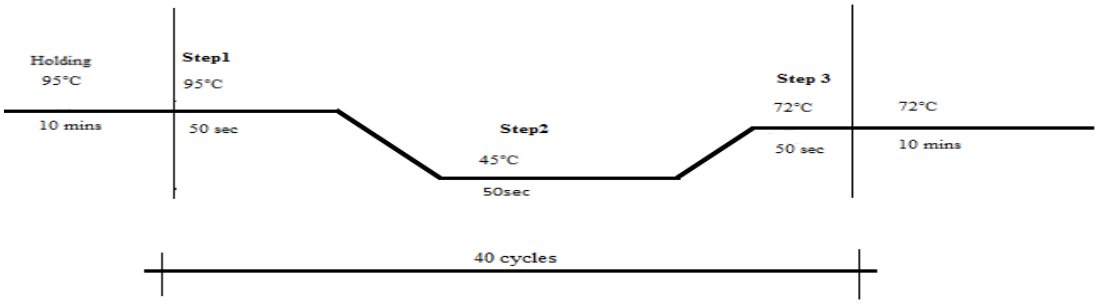


Figure 3. Conditions for PCR reaction

4.4. Gel Electrophoresis

After PCR completed 1.4% of agarose gel was prepared. For this aim, 0.7 g of agarose was dissolved in 50 ml 1X TAE buffer and boiled in microwave oven for 5 min. The solution was then cooled under tap water, 4 µl of Ethidium bromide (10 mg/ml) was added to the solution. The gel was then transferred to the casting tray combs were placed to form sample wells. The gel was then left for cooling at room temperature for 30 minute. Then the comb was removed and 1X TAE buffer was poured on gel to cover. 10 µl of each samples were loaded into the wells after they were mixed with 1µl of 6x loading dye. The gel was run with 90V constant current for 30 minutes. Finally, it was visualized under the UV light, the good bands giving samples were then selected and sent for Gene sequencing.

4.5.Gene Sequencing

A total of 70 cattle samples and 20 sheep samples were selected after gel electrophoresis and sent to the gene sequencing company (BM Labosis, Turkey).

4.6. Data analysis by using bioinformatics tools

Once we acquired raw gene sequences of the selected samples, some bioinformatics tools have been used for the analysis of data. “Finch TV” has been used for viewing the sequencing data. “BLAST” have been used to compare the sequences of our samples with the database and to find the genotype of each sample. The sequence ends were trimmed by comparing the published sequences using “BLAST” search. The trimmed sequences were then added to “CLC Sequence viewer 8” program. The

alignment was performed with the published reference sequences and out group sequences retrieved from NCBI Pubmed. *Echinococcus granulosus* and *E. canadansis* were used as reference sequences, whereas *E. multilocularis*, *E. equinus*, *E. ortleppi* and *E. felidis* were used as outgroup sequences. Through alignment the circular phylogram have been generated in order to show the phylogenetic relationships of the samples.

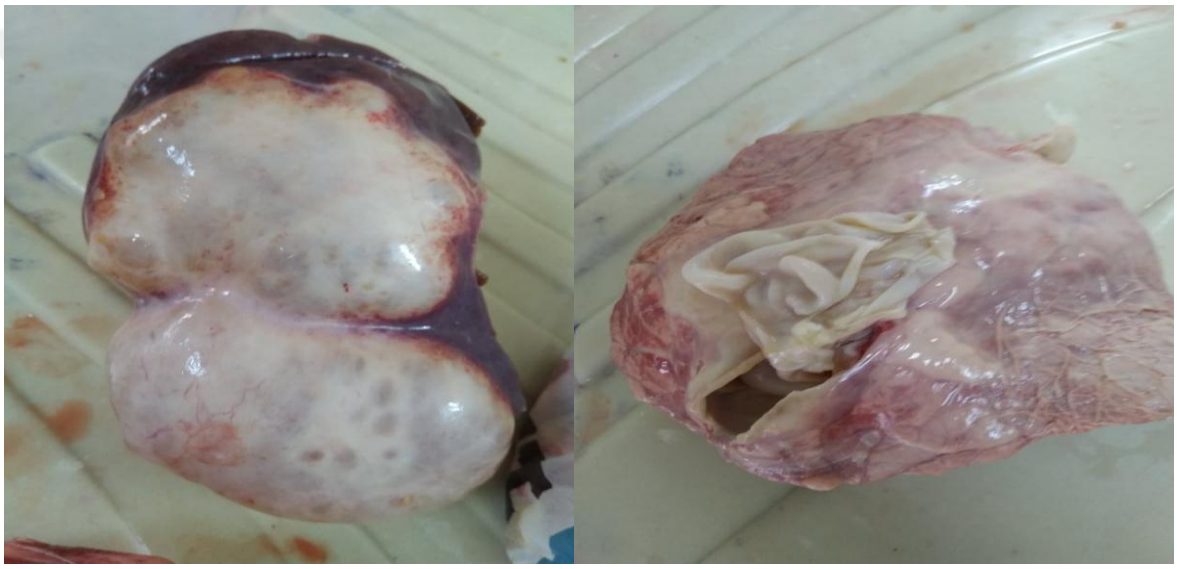
4.7. Haplotype Analysis

Haplotype analysis have been performed on cattle as well as sheep samples by using PopArt haplotype network 1.7. The method used for the analysis is "Minimum spanning Network"

5. RESULTS

5.1. Sample selection criteria

A total of 128 hydatid cysts were collected from lungs and liver of 67 animals (46 cattle, 21 sheep). The hydatid cysts were then dissected and germinal layers were collected for the further studies.



A **B**

Figure 4.Showing the hydatid cysts with germinal layer. A: Sheep liver hydatid cysts contain cysts fluid, B: Dissected lung hydatid cysts and showing the germinal layer

Samples were selected in two steps

1. Those samples which were completely calcified were excluded.
2. There were few collected samples which were partially calcified and were also filled with water fluid and the germinal layer of such samples can easily be

observed as shown in part A of the Figure 5. These germinal layers (partial calcified) and the germinal layers from fluid filled hydatid cysts (non-calcified) were collected and processed for gDNA extraction. And after gDNA extraction, based on PCR results, samples were selected for gene sequencing.

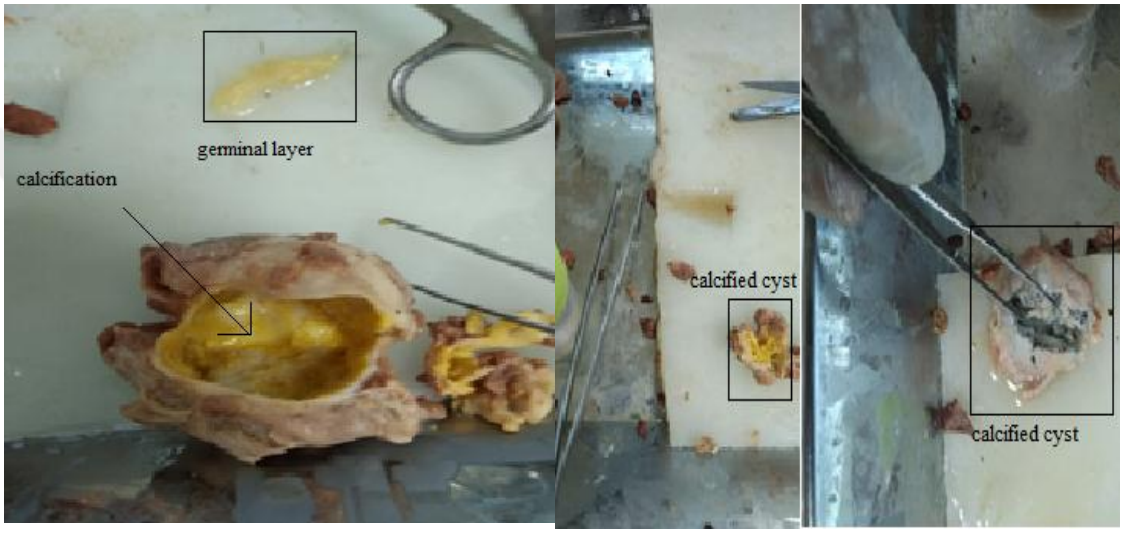


Figure 5. Showing the calcified hydatid cysts. A: Dissected hydatid cyst which was not fully calcified and thus the germinal layer of such samples were collected for further process, B: Cysts which were completely calcified and thus excluded from the study at start.

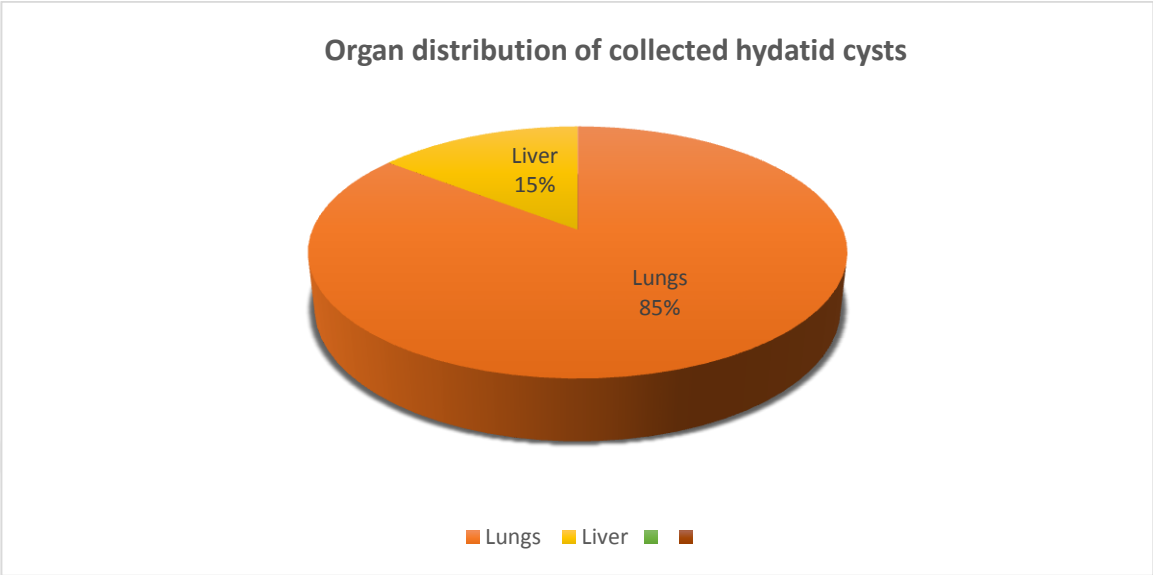


Figure 6. Pie-graph showing the percentage of collected hydatid cyst samples in organs.

The percentage of cyst infection in organs was described in pie graph, 85% of the cyst infection found in lungs where as 15 % of cysts found in liver.

5.2. PCR Results

The gene of interest of our study is the 446 bp region of CO1. The amplified 446 bp region of CO1 can be observed in the Figure 7. The 100 bp DNA ladder was used as a marker in the gel, the positive control used in the study showing the 446 bp region of CO1 and the negative control showed no contamination in PCR reaction.

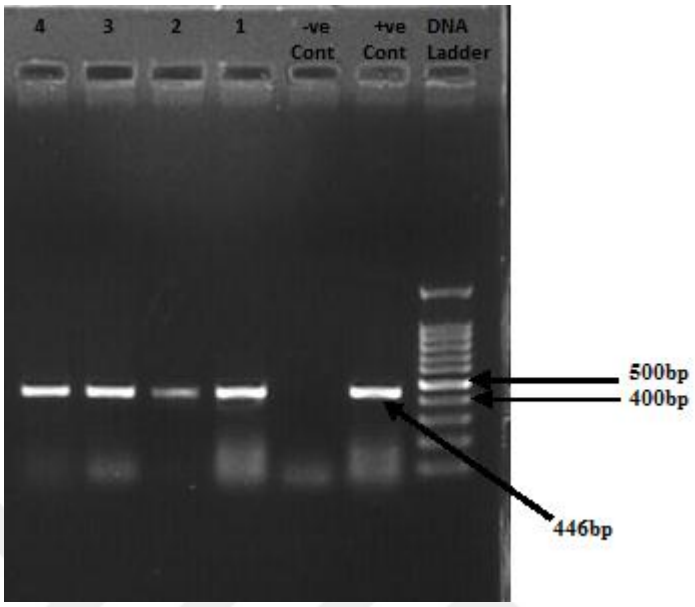


Figure 7. PCR results.Lane one is showing the 100 bp DNA ladder, lane two is showing the positive control of 446 bp of Echinococcus sample and lane three is showing the negative control and the remaining lanes are showing the samples.

The results of the PCR reactions were analyzed on the basis of following criteria;

PCR bands were divided into four categories

1. Very good bands
2. Good bands
3. Weak bands
4. No bands

Samples with no bands and weak bands were excluded from the study whereas the PCR products of samples with good and very good bands were stored at -20° for further study. At the end 90 samples were selected 20 sheep and 70 cattle and were sent to company for Gene Sequencing.

The Table 2 is showing the samples which were being selected on the basis of PCR results.

Table 2. Samples with good PCR results and sequenced

Sr No	Samples	Animals	Organs	PCR Results
1	C1	Cattle 1	Lungs	+++
2	C2			+++
3	C3			+++
4	C4			+++
5	C5			+++
6	C6	Cattle 2	Lungs	++
7	C7	Cattle 3	Lungs	++
8	C8			++
9	C9	Cattle 4	Lungs	+++
10	C10			++
11	C11			++
12	C12	Cattle 5	Liver	++
13	C13			++
14	C14			++
15	C15			++
16	C16			++
17	C17			++
18	C18	Cattle 5	Lungs	++
19	C19			++
20	C20			++
21	C21	Cattle 6	Lungs	++
22	C22			++
23	C23			++
24	C24	Cattle 7	Lungs	++
25	C25			++
26	C26			++
27	C27	Cattle 8	Lungs	+++
28	C28			++
29	C29			+++
30	C30			++
31	C31			++

32	C32	Cattle 8	Liver	++
33	C33			++
34	C34			++
35	C35			++
36	C36			++
37	C37			++
38	C38	Cattle 9	Lungs	++
39	C39			++
40	C40			++
41	C41	Cattle 10	Lungs	++
42	C42			++
43	C43	Cattle 11	Lungs	+++
44	C44	Cattle 12	Lungs	++
45	C45			++
46	C46	Cattle 13	Lungs	++
47	C47			++
48	C48	Cattle 14	Lungs	++
49	C49	Cattle 15	Liver	++
50	C50			++
51	C51	Cattle 16	Lungs	++
52	C52	Cattle 17	Lungs	++
53	C53	Cattle 18	Lungs	++
54	C54	Cattle 19	Liver	++
55	C55	Cattle 20	Liver	++
56	C56	Cattle 21	Lungs	++
57	C57	Cattle 22	Liver	++
58	C58	Cattle 23	Liver	++
59	C59	Cattle 24	Lungs	++
60	C60	Cattle 25	Lungs	++
61	C61	Cattle 26	Lungs	++
62	C62	Cattle 27	Lungs	++
63	C63	Cattle 28	Lungs	++
64	C64	Cattle 29	Lungs	++
65	C65	Cattle 30	Lungs	++
66	C66	Cattle 31	Lungs	+++
67	C67	Cattle 32	Lungs	+++
68	C68	Cattle 33	Lungs	+++
69	C69	Cattle 34	Lungs	+++

70	C70	Cattle 35	Lungs	++
71	S1	Sheep 1	Lungs	++
72	S2	Sheep 2	Lungs	++
73	S3	Sheep 3	Lungs	++
74	S4	Sheep 4	Lungs	++
75	S5	Sheep 5	Liver	++
76	S6	Sheep 6	Lungs	++
77	S7	Sheep 7	Lungs	++
78	S8	Sheep 8	Lungs	++
79	S9	Sheep 9	Lungs	++
80	S10	Sheep 10	Lungs	++
81	S11	Sheep 11	Lungs	++
82	S12	Sheep 12	Lungs	++
83	S13	Sheep 13	Lungs	++
84	S14	Sheep 14	Lungs	++
85	S15	Sheep 15	Lungs	+++
86	S16	Sheep 16	Lungs	++
87	S17	Sheep 17	Lungs	++
88	S18	Sheep 18	Lungs	++
89	S19	Sheep 19	Lungs	+++
90	S20	Sheep 20	Lungs	+++

"++" indicates samples with good bands, "+++" indicates samples with very good bands.

In our study multiple organ infections have been found in two cattle (cattle 5 and cattle 9) as shown in Table 2. The cysts were in liver and lungs.

5.3.Gene Sequencing

As shown in Table 2, a total of 90 samples were sent for gene sequencing, out of them 5 samples were not sequenced properly, upon re-sequencing of those samples we couldn't get good results so those samples have been excluded. The alignment was performed on all sequences and also on samples of same cattle. The results have shown the single nucleotide polymorphism in some samples of same cattle

5.4.Alignment of the Sequence Results

All the sequences of 85 samples were analyzed along with the reference sequences of *E. granulosus s.s*. and *E. Canadensis, E. multilocularis, E. equinus, E. ortleppi* and *E. felidis* were used as outgroups retrieved from the GenBank in order to analyze the phylogeny of the samples and their relationship. The results have shown the predominance of *E. granulosus s.s.* in all the samples except one sheep sample (S2) which has shown resemblance with the *E. canadansis.* The presence of camel strain in sheep is reported for the first time in Turkey. However we have observed mutations (C-T and T-C substitution) at different positions within the sequences of *E. granulosus s.s.* Thus we performed alignments on samples which were taken from same cattle and also generated the haplotypes of cattle and sheep samples separately in order to analyze our results in more detail.

The Figure 8 is showing the alignment of 85 samples with using of "CLC sequence viewer

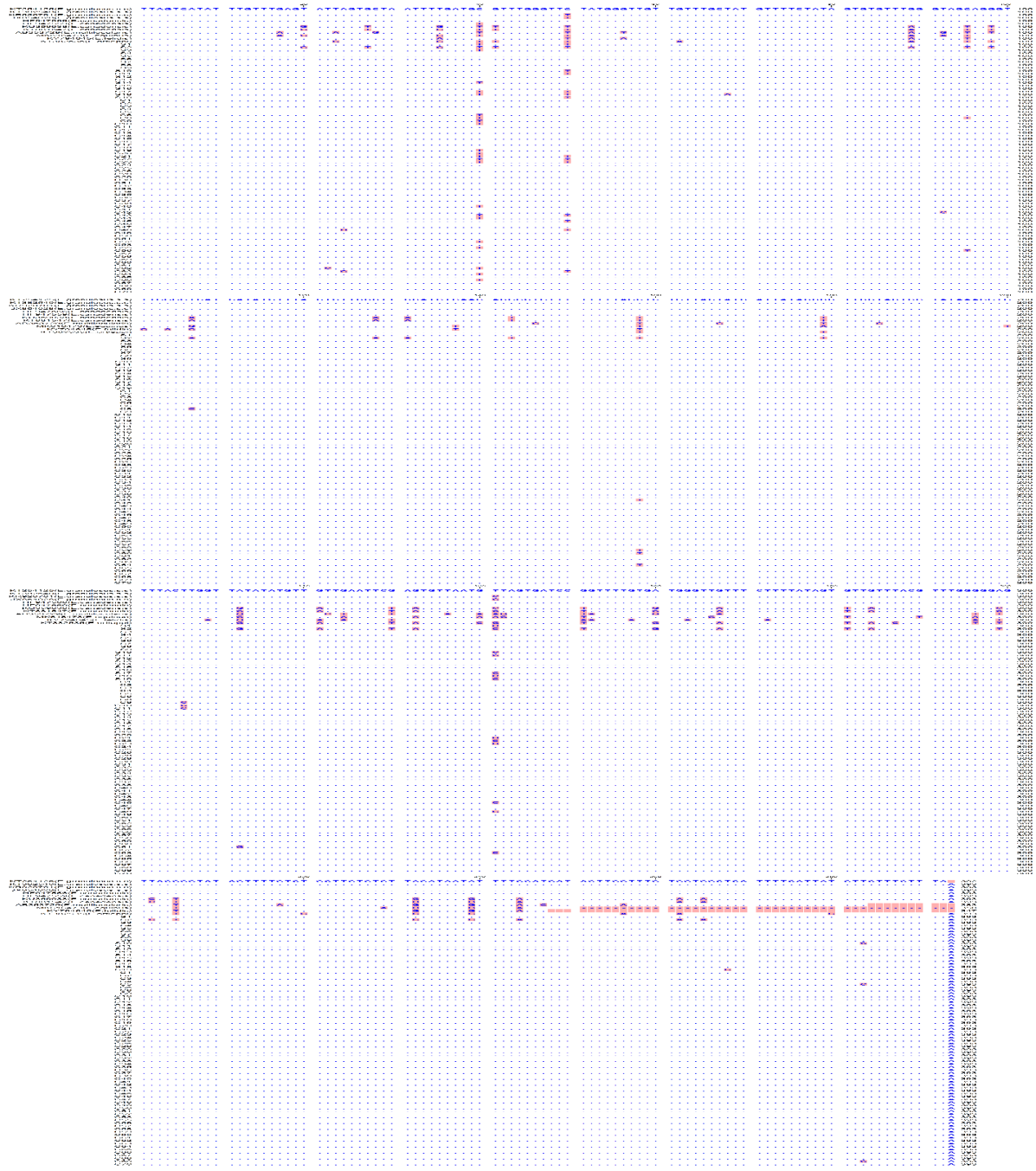


Figure 8. Alignment of the sequenced samples. The following reference sequences were used for alignment: KT382540 (*E.granulosus.s.s*), KR920701 (*E.granulosus.s.s*), KT254125 (*E.granulosus.s.s*), KT881547 (*E.canadensis*), KU359038 (*E.canadensis*), KT382535 and outgroup references are KT382535 (*E.ortleppi*), AB353729 (*E.multilocularis*), MK616473 (*E.equinus*), KY794645 (*E.felidis*).

5.5.Phylogenetic relationship

Circular phylogram was generated through alignment of the sequences showing the phylogenetic relationship of samples with their reference sequences and outgroups retrieved from BLAST search.

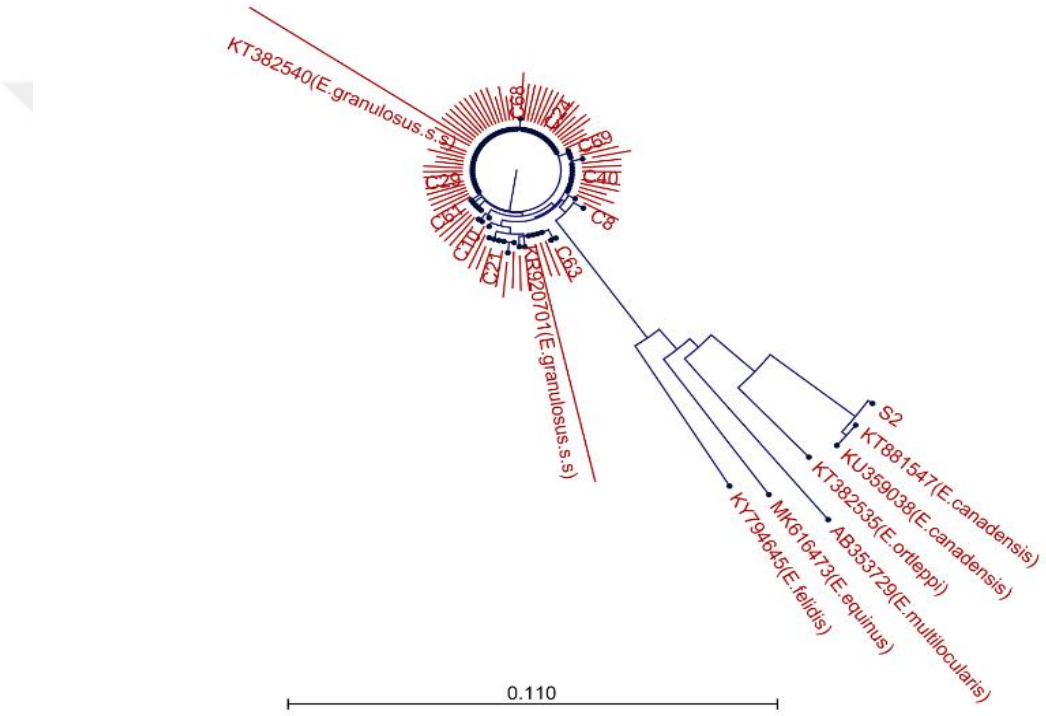


Figure 9: Circular phylogram showing the relationships between samples. References used for the sequences are KT382540 (*E.granulosus. s.s*), KR920701 (*E.granulosus. s.s*), KT254125 (*E.granulosus.s.s*), KT881547 (*E.canadensis*), KU359038.1 (*E.canadensis*), and outgroup references are KT382535 (*E.ortleppi*), AB353729 (*E.multilocularis*), MK616473 (*E.equinus*), KY794645 (*E.felidis*)

The Figure 9 shows the phylogenetic relationship of samples. The BLAST search of the S2 sequence has shown G6 genotype of *E. canadensis* and as shown in Figure 8 and Figure 9. S2 shares same branch with *E. canadensis* and thus showed the close

relatedness with *E. canadensis*. This close relatedness with *E. canadensis* shows the presence of G6 strain in the sheep isolate.

5.6.Alignment of same organ hydatid cysts of cattle

Three lung samples were collected from same cattle. Results have shown the presences of *E. granulosus s.s.* in the samples. However, a single nucleotide polymorphism was observed in 40th nucleotide in a sample (C11) of cattle 4, where there was T-C substitution occurred as shown in Figure 10.

Figure 10: Sequence comparison of same organ hydatid cysts of cattle 4

In our study multiple organ infection have been found. No differences have been observed in the nucleotides of liver samples of cattle 5. Although, three samples collected from lungs of same cattle showed the presence of *E. granulosus s.s.*, single nucleotide polymorphism was observed in 40th nucleotide as shown in Figure 11.

Figure 11. Sequence comparison of lungs hydatid cysts of cattle 5.

The comparison of liver and lungs samples of same cattle (cattle 5) have shown C-T substitution at 40th nucleotide of C19 and C20 as shown in Figure 12. C19 and C20 are the sequences of lungs isolates which shows the difference of one nucleotide with the sample of the same lungs as well as with liver samples of same cattle.

Figure 12. Sequence comparison between lungs and liver hydatid cysts of Cattle 5.

The samples of Cattle 9 have shown C-T substitution at 40th nucleotide as shown in Figure 13.

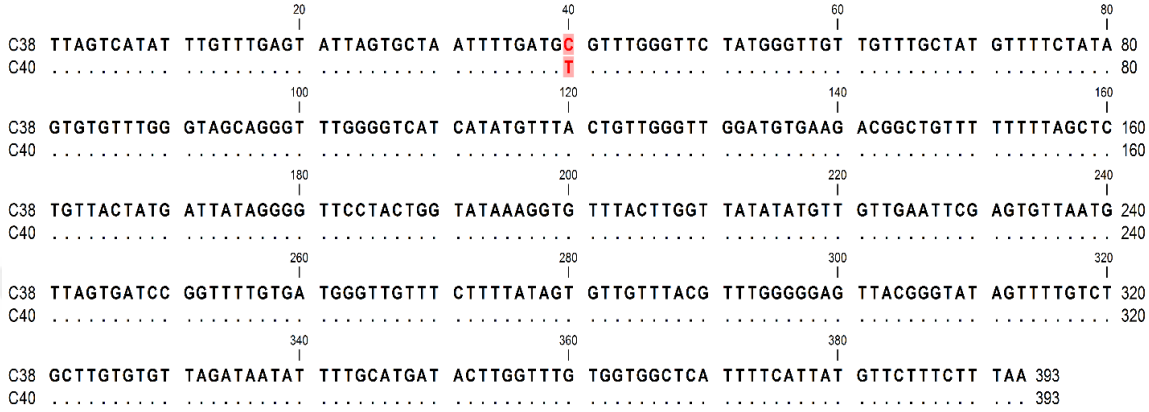


Figure 13. Sequence comparison of same organ hydatid cysts of cattle 9.

The samples of cattle 10 have shown *E. granulosus s.s.* genotype, however mutations at two points have been observed, T-C substitutions at nucleotide 92 and 158 as shown in Figure 14.

Figure 14. Sequence comparison of same organ hydatid cysts of cattle 10

Samples of cattle 12 has shown *E. granulosus s.s.*, however mutations at three points were observed in the nucleotides, T-C substitution at 40th and 241th nucleotides and C-T substitution at 50th nucleotide as shown in the Figure 15.

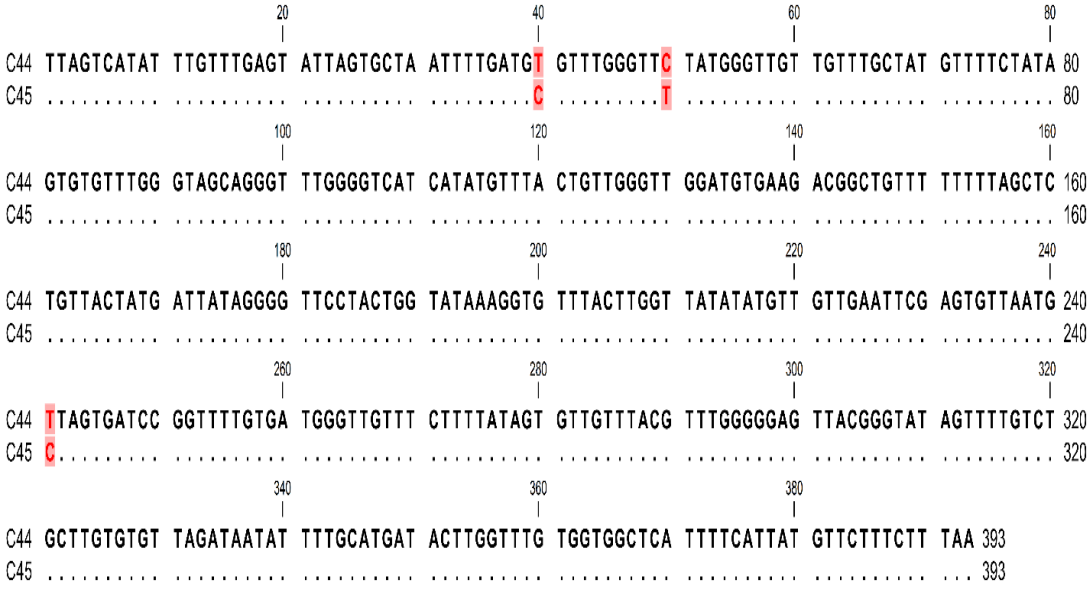


Figure 15. Sequence comparison of same organ hydatid cysts of Cattle 12

5.7. Haplotype analysis of cattle samples

A total of 16 haplotypes of *E. granulosus s.s* have been detected in 66 cattle isolates and the results were presented in Figure 16.

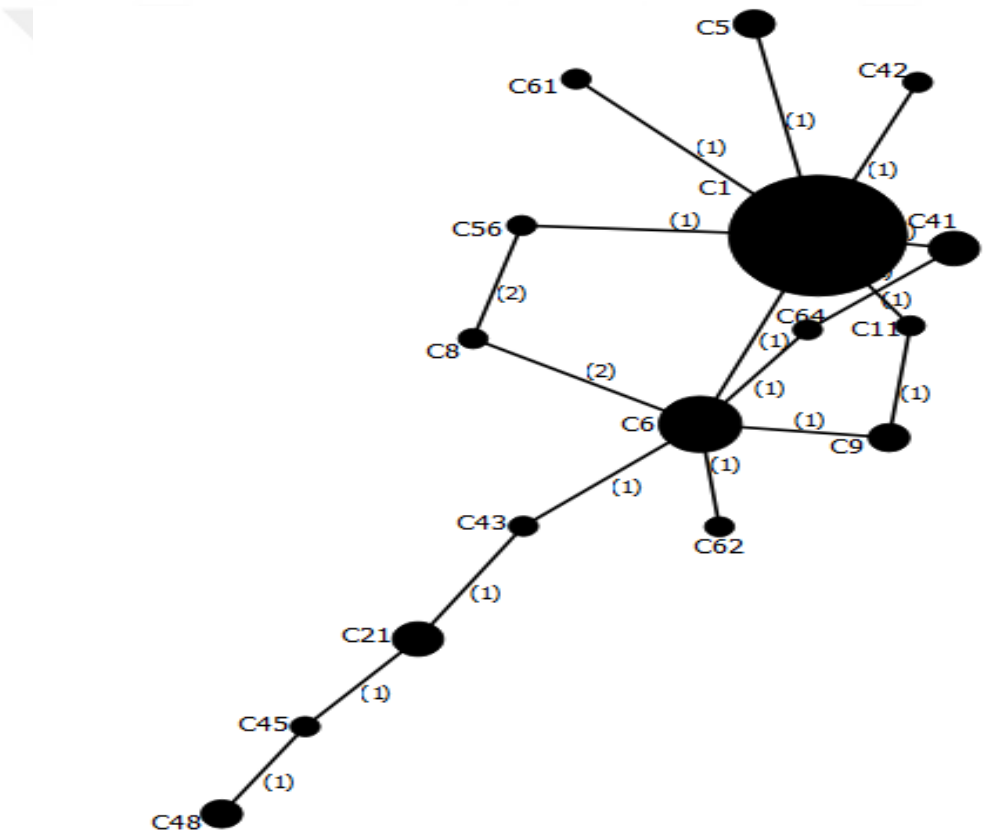


Figure 16. The appearance of mt-CO1 haplotypes of cattle isolates of *E. granulosus s.s.* The size of the circles is related to the haplotype frequency. Small circles indicate additional mutational areas. The numbers in the figure indicates the number of mutations.

5.8.Haplotype Analysis of sheep samples

The haplotype analysis of sheep isolates has shown 7 different haplotypes of *E.granulosus s.s.* with one or two number of mutations and the results were described in the Figure 17.

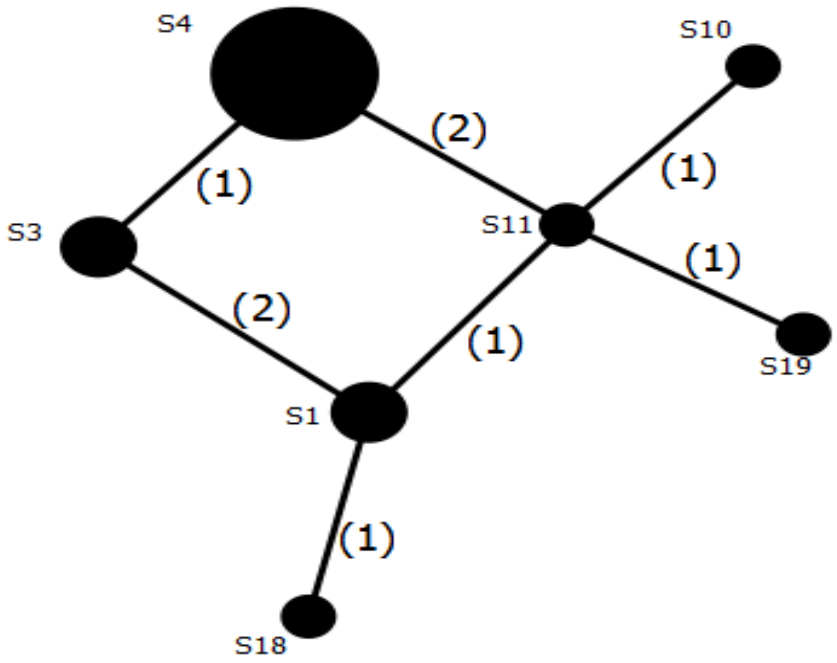


Figure 17. The appearance of mt-CO1 haplotypes of sheep isolates of *E. granulosus s.s.* The size of the circles is related to the haplotype frequency. Small circles indicate additional mutational areas. The numbers indicating the number of mutations.

6. DISCUSSION

Echinococcosis which is a life threatening zoonotic infection, recognized as 17 neglected tropical disease in the world by WHO, caused by the larval stage of cestodes species of genus Echinococcus and is responsible for significant economic as well as medical losses each year (1; 6; 161). *Echinococcus granulosus* expresses variety of phenotypic variations with respect to host specificity, adult and larval stage morphology, antigenicity, pathogenicity and biochemical composition and genetic makeup of nucleic acid sequences are responsible for the appearance and differentiation of genetic variants, genotypes and strains within Echinococcus and some of them have been given status of new species (4; 5; 89). DNA based methods are more suitable than conventional approaches for determining the genetic identity of particular organism. Another advantage of DNA based methods is that the DNA sequencing and comparison allows direct examination of the genome independently of environmental factors and otogenic influences. The divergence level can easily be quantitating and the range of nucleotide characters for phylogenetic analysis is practically unlimited. In studies conducted to determine the genotypes of *E. granulosus* by using mitochondrial region of CO1, DNA based methods have been used which includes PCR reaction and gene sequencing of selected samples (75). Most of the Middle East countries have been considered as the main foci of CE infection for both humans and animal. Though metacestodes of *E. granulosus* is being reported in almost all countries of Middle East but Iraq, Iran and Turkey have more prevalence as compared to other countries (162). For the molecular

classification and to determine the genotypes of *E. granulosus* mitochondrial as well as nuclear genomes have been used, however for phylogenetic analysis of *E. granulosus* among closely related species, mt-DNA has been more efficient as compare to nuclear genomes because of the large datasets and rapid sequence evolution (163). The mt-CO1 can be used as a significant evolutionary marker for the distinction of Intra- and Interspecific variants (9).

Generally, *E. granulosus s.s.* are considered as predominant genotype in CE infections worldwide (8; 164). In the current study, 128 hydatid cysts were collected from 67 animals (46 cattle, 21 sheep) from a local slaughterhouse (EL-KAS) in Elazig. Our study has showed the predominance infection in lungs 85% as compared to liver 15% which is in agreement with the previous studies, one of the study was done on slaughtered goats from various municipal abattoirs in Oman, the study showed the predominance of infection in lungs 53.4% (165), another study conducted in Oman which showed the high prevalance in lungs 82.9 % (166). Moreover in a recent study, 23 out of 30 cysts found in lungs (160). The predominance of infection in lungs can be explained by the fact that the *Echinococcus* oncospheres migrate to the lungs and lungs possess great number of capillaries sites than any other organ (167). Moreover the viability rate of the protoscoleces established in the lungs was reported significantly higher 68.8% than any other organs (165).

Our results have shown that 84 out of 85 samples (66 cattle, 19 sheep) were detected as *E. granulosus s.s,* which is in agreement with recent studies. In a recent study out of 12 sheep isolates and 10 human isolates of *E. granulosus*, 19 isolates have

shown the presence of *E. granulosus s.s.* (91). Another study has been conducted on 120 isolates from two provinces (Elazig and Erzincan) out of them 114 isolates have shown the presence of *E. granulosus s.s*. through 12S rRNA-PCR while 6 samples of mt-CO1 gene has shown the presence of *E. granulosus s.s* in all 6 samples (160). Moreover in another study has been conducted on 42 human isolates, 13 cattle and 3 sheep isolates in the Thrace region of Turkey, the G1 genotype (sheep strain) have been found in all cattle and sheep samples, however in human isolates G1 and G7 genotypes were identified (169). However in the current study one sheep sample has shown the presence of *E. canadensis* (G6 genotype) and upon CO1 analysis 100% homology was found with *E. canadensis G6* reference sequence, which is reported for the first time in Turkey. G6 genotype of *E. canadensis* has been reported in sheep and cattle in Sudan (110), although the prevalence of this genotype is comparatively low in sheep and cattle as compared to camel (110; 170). The study conducted in Turkey on 70 FFPE tissue samples of humans, the results have shown the presence of G6 genotype in two of the human isolates which conclude the fact that there is presence of (camel strain) in the region (159). Echinococcus spp has unique reproduction system as the adult worms are hermaphrodites and their asexual reproduction occurs at larval stage, this unique process of reproduction is responsible for the variations at genus and specie level because of self-fertilization following the amplification by asexual reproduction which results in mutations (171). The study showed the presence of two different haplotypes in a single intermediate host (172). In our study sequence analysis has also been performed on samples of same cattle which showed the single nucleotide polymorphism within the samples of same animals. In this study we have also found multiple occurrence hydatid

cysts in same organ in two cattle (cattle 5 and cattle 8) shown in Table 2, moreover upon comparing the samples of lung and liver of same cattle we have found the substitution of C-T in two lungs samples (C18, C19) of cattle 5 at nucleotide number 40 as shown in Figure 12. Studies have shown the infection in multiple occurrence, in some cases 20-40% of individuals might have multiple cysts which may involve multiple organs (168).The substitution of C-T at 40^{th} nucleotide of the samples C9, C11 and C12 of cattle 4; C18, C19 and C20 of cattle 5 and C38 and C40 of cattle 9 have been observed trough alignment. Whereas in C41 and C42 samples of cattle 10 there were substitutions of C-T at two points at 92^{nd} and 158^{th} position of nucleotides, and in C44 and C45 of cattle 12 the substitutions have been found at third points of 40^{th}, 50^{th} and 241^{st} nucleotide, which shows the presence of different variants of *E. granulosus s.s.* within same animal and the presence different variants in single intermediate host shows the out-crossing between different adult worms may occur in same definitive host.

We have also generated the haplotypes of *E. granuslosus s.s.* by using sequence alignment of sheep and cattle samples separately. There were 16 haplotypes of *E.granulosus s.s.* found in 66 cattle samples with the mutations at either one or two points. However in sheep samples 7 haplotypes of *E. granulosus s.s.* have been found with mutations either at one point or two. Study has reported extremely high haplotype diversity in G1 genotype as reported 171 haplotypes out of 212 samples have found (173). Many other studies have reported excessive genetic diversity of *E. granulosus s.s.* in various parts of the world based on shorter sequence lengths (90; 174-177).CO1 gene is considered to be the most appropriate candidate for investigating the genetic diversity

of *E. granulosus s.s.* at intraspecific level (178-181). Study has reported the presence of *E. granulosus s.s.* (G1-G3) in Eastern European and Italian population and confirmed 21 haplotypes in Eastern Europe and four haplotypes in Italy (172).

In conclusion, 85 isolates (19 sheep, 66 cattle) were sequenced out of them 84 were identified as *E. granulosus s.s.* and polymorphism within *E. granulosus s.s.* has been observed in not only samples of different animals but also the isolates collected from same cattle, moreover multiple organ infections in two cattle have also been detected in both lungs and liver, and samples of one of the cattle (both lungs and liver) also showed the C-T substitution. The presence of polymorphic sites within the samples of same animal indicates the chance of crossing over between worms in definitive hosts, however more study is needed in this section. We have found 16 haplotypes of *E. granulosus s.s.* within cattle isolates and 7 haplotypes in sheep samples. However, we have found G6 genotype of *E. canadensis* in one of the sheep sample which is identified for the first time in sheep in Turkey. Although previous study has reported the presence of G6 in two human isolates, this indicates the presence of this genotype in livestock as well as in humans in this region.

7. REFERENCES

1. Iván N, Higuita A, Brunetti E, Cindy McCloskey. Cystic echinococcosis. J Clin Microbiol 2016; 54 (3): 518-523.
2. Angela M, Restrepo C, Yang YR, McManus DP et al. Spatiotemporal patterns and environmental drivers of human echinococcoses over a twenty-year period in Ningxia Hui Autonomous Region,China. Parasites Vectors 2018; 11: 108.
3. Hüttner M, Nakao M, Wassermann T et al. Genetic characterization and phylogenetic position of Echinococcus felidis (Cestoda: Taeniidae) from the African lion. Int J Parasitol 2008; 38: 861–86.
4. Xiao N, Qiu J, Nakao M et al. *Echinococcus shiquicus* n. sp., a taeniid cestode from Tibetan fox and plateau pika in China. Int J Parasitol 2005; 35: 693-701.
5. Nakao M, Yanagida T, Konyaev S et al. Mitochondrial phylogeny of the genus Echinococcus (Cestoda: Taeniidae) with emphasis on relationships among *Echinococcus canadensis* genotype. Parasitol 2013; 140: 1625-1636.
6. Agudelo Higuita NI, Brunetti E. Cystic Echinococcosis. J Clin Microbiol 2016; 54: 518–23.
7. Abushhewa MH, Abushhiwa MH, Nolan MJ et al. Genetic classification of Echinococcus granulosus cysts from humans, cattle and camels in Libya using mutation scanning-based analysis of mitochondrial loci. Mol Cell Probes 2010; 24: 346-51.

8. Alvarez Rojas CA, Romig T, Lightowlers MW. Echinococcus granulosus sensu lato genotypes infecting humans-review of current knowledge. Int J Parasitol 2014; 44 (1): 9–18.
9. Nakao M, Lavikainen A, Yanagida T, Ito A. Phylogenetic systematics of the genus Echinococcus (Cestoda: Taeniidae). Int J Parasitol 2013; 43: 1017-1029.
10. Thompson RC. The taxonomy, phylogeny and transmission of Echinococcus. Exp. Parasitol. 2008; 119: 439–446.
11. Thompson RC, Boxell AC, Ralston BJ et al. Molecular and morphological characterization of Echinococcus in cervids from North America. Parasitology. 2006; 132: 439-447.
12. Romig T, Ebi D, Lato MW. Taxonomy and molecular epidemiology of Echinococcus granulosus sensu lato. Vet Parasitol 2015; 213: 76-84.
13. Pawlowski I, Eckert J, Vuitton D. Echinococcosis in humans: clinical aspects, diagnosis and treatment. In: Eckert J, Gemmell M, Meslin F-X, Pawlowski Z, eds. WHOI/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Paris : s.n. 2001; 20-71.
14. Galeh TM, Spotin A, Mahami-Oskouei M et al. The seroprevalence rate and population genetic structure of human cystic echinococcosis in the Middle East: A systematic review and meta-analysis. Int J Surg 2018; 51: 39-48.
15. Rinaldi F, Brunetti E, Neumayr A, Maestri M, Goblirsch S, Tamarozzi F. Cystic echinococcosis of the liver: A primer for hepatologists. World J Hepatol 2014; 6: 293-305.

16. Gattu M, Saiyad Jameer Bhasha T. A study on profile of hydatid cyst of liver presented at a tertiary care hospital. Int Surg J 2017; 4 (1): 121-124.

17. Sabouni F, Ferdosian F, Mamishi S et al. Multiple organ involvement with hydatid cysts. Ir J Parasitol 2010; 5 (2): 65-70.

18. Pourseif M, Moghaddam G, Saeedi N et al. Current status and future prospective of vaccine development against Echinococcus granulosus. Biologicals 2017; 51: 1-11.

19. D'Alessandro A, Rausch RL. New aspects of neotropical polycystic (*Echinococcus vogeli*) and unicystic (*Echinococcus oligarthrus*) echinococcosis. Clin. Microbiol Rev 2008 ; 21 (2): 380-401.

20. Eckert J, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. Clin Microbiol Rev 2004 ; 17 (1): 107-135.

21. Moro P, Schantz PM. Echinococcosis: a review. Int J Infect Dis 2009; 13 (2): 125-133.

22. Haridy FM, Ibrahim BB. Sheep-dog-man. The risk zoonotic cycle in hydatidosis. J Egypt Soc Parasitol 2000; 30(2): 423-429.

23. Lahmar S, Debbek H, Zhang LH et al. Transmission dynamics of the Echinococcus granulosus sheep-dog strain (G1 genotype) in camels in Tunisia. Vet Parasitol 2004; 121(1-2): 151-156.

24. Cornejo-Juárez, Espinosa-Altamirano A, Ibarra-del-Río M, Pacheco-Bravo I. Cystic echinococcosis: a disease mimicking cancer in a non-endemic country report of two cases. West Indian Med J 2013; 62 (3): 266-269.

25. Eckert J, Gemmell MA, Meslin F-X, Pawlowski ZS. Geographic distribution and prevalence. . Schantz P, Gasser R Eckert J. Paris : s.n World Organisation for Animal Health 2001: 100-41.

26. Eckert J, Gemmell M, Meslin A et al. A Public Health Problem of Global Concern, WHO/OIE Man Echinococcosis Humans Anim. 2001.

27. Ito A, Urbani C, Jiamin Q et al. Control of echinococcosis and cysticercosis: a public health challenge to international cooperation in China. Acta Trop 2003; 86: 3-17.

28. McManus DP. The molecular epidemiology of Echinococcus granulosus and cystic hydatid disease. Trans R Soc Trop Med Hyg 2002; 96: 151-57.

29. Thompson RC. Aetiology: parasites and life-cycles. Gemmell M, Meslin F-X, Pawlowski Z, Eckert J. WHOI/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. Paris : s.n., 2001: 1-19.

30. Castrodale LJ, Beller M, Wilson JF et al. Two atypical cases of cystic echinococcosis (*Echinococcus granulosus*) in Alaska. Am J Trop Med Hyg 1999 ; 66: 325-37.

31. Mcmanus DP, Zhang W, Bartley PB. Echinococcosis. Lancet 2003 ; 362(9392): 1295-304.

32. Menghebat L, Jiang L. A retrospective survey for surgical cases of cystic echinococcosis in the Xinjiang Uygur Autonomous Region, PRC (1951–90). Chai J Liu F, Andersen F. Compendium on cystic echinococcosis with special reference to the XinjiangUygur Autonomous Regi, in Provo. 1993: 135-45.

33. Gottstein B. Molecular and immunological diagnosis of echinococcosis. Clin Microbiol Rev 1992; 5(3): 248-261.

34. Lightowlers M, Gottstein W. Echinococcosis/hydatidosis: antigens, immunological and molecular diagnosis. R. C. A. Thompson and A. J. Lymbery. The biology of echinococcus and hydatid disease. 1995: 355-410.

35. Jenkins DJ, Rickard MD. Specificity of scolex and oncosphere antigens for the serological diagnosis of Taeniid cestode infections in dogs. Aust Vet J 1986; 63(2): 40-42 .

36. Ito A. Serologic and molecular diagnosis of zoonotic larval cestode infections. Parasitol Int 2002; 51(3): 221-35.

37. Poretti D, Felleisen E, Grimm F. Differential immunodiagnosis between cystic hydatid disease and other cross-reactive pathologies. Am J Trop Med Hyg 1999; 60(2): 193-98.

38. Zhang W, Jun Li, Donald P, McManus D P. Concepts in immunology and diagnosis of hydatid disease. Clin Microbiol Rev 2003; 16(1): 18-36.

39. Kittelberger R, Reichel M, Jenner J. Evaluation of three enzymelinked immunosorbent assays (ELISAs) for the detection of serum antibodies in sheep infected with Echinococcus granulosus. Vet Parasitol 2002; 110(1-2): 57-76.

40. Deplazes P, Eckert J. Diagnosis of the Echinococcus multilocularis infection in final hosts. Appl Parasitol 1996 ; 37(4): 245-52 .

41. Gottstein B, Deplazes P, Eckert J. Serological (Em2-ELISA) and parasitological examinations of fox populations for Echinococcus multilocularis infections. Zentralbl 1991; 38(3): 161-8.

42. Craig P, Macpherson C, Watson-Jones D. Immunodetection of Echinococcus eggs from naturally infected dogs and from environmental contamination sites in settlements in Turkana, Kenya. Trans R Soc Trop Med Hyg 1988 ; 82: 268-74 .

43. Deplazes P, Gottstein B, Eckert J, Jenkins D. Detection of Echinococcus coproantigens by enzyme-linked immunosorbent assay in dogs, dingoes and foxes, Parasitol Res 1992 ; 78: 303-08 .

44. Horton RJ. Albendazole in treatment of human cystic echinococcosis: 12 years of experience. Acta Trop 1997; 64(1-2): 79-93.

45. Franchi C, DiVico B. Long-term evaluation of patients with hydatidosistreated with benzimidazole carbamates. Clin Infect Dis 1999 ; 29: 304-09 .

46. Liu Y. Continuous or intermittent treatment with albendazole?. Arch Int Hidatid 1997; 32: 171-73.

47. Gil-Grande L, Rodriguez-Caabeiro F, Prieto J. Randomised controlled trial of efficacy of albendazole in intra-abdominal hydatid disease. Lancet 1993; 342(8882): 1269-72 .

48. Mohamed A, Yasawy M. Combined albendazole and praziquantel versus albendazole alone in the treatment of hydatid disease. Hepatogastroenterology 1998; 45(23): 1690-94 .

49. He YB, Yao G, Tuxun T et al. Efficacy of radical and conservative surgery for hepatic cystic echinococcosis: A meta-analysis. Int J Clin Exp Med 2015 ; 8(5): 7039-7048.

50. Lightowlers MW, Lawrence SB, Gauci CG, et al. Vaccination against hydatidosis using a defined recombinant antigen. Parasite Immunol 1996; 18(9), 457-462.

51. Huang, Dang Z, Suzuki Y, Horiuchi T, Yagi K, Kouguchi H. Analysis on gene expression profile in oncospheres and early stage metacestodes from Echinococcus multilocularis. PLoS Negl Trop Dis 2016 ; 10(4): e0004634.

52. Varcasia A, Canu S, Lightowlers MW, Scala A, Garippa G. Molecular characterization of Echinococcus granulosus strains in Sardinia. Parasitol Res 2006; 98(3): 273-277 .

53. Morariu S, Marshall W, Lightowlers M et al. Utilization of EG95 vaccine for sheep immunization against cystic echinococcosis in Romania. Sci Parasitol 2010 ; 11: 29-34.

54. Lightowlers M, Jensen O, Fernandez E. Vaccination trials in Australia and Argentina confirm the effectiveness of the EG95 hydatid vaccine in sheep. Int J Parasitol 1999 ; 29(4): 531-34 .

55. Abuladze K. TAENIATA of animals and man and diseases cause by them. A.K.I. Skrjabin. Essentials of Cestodology. 1964 , IV.

56. Tappe D, Kern P, Frosch M, Kern P. A hundred years of controversy about the taxonomic status of Echinococcus species. Acta Trop 2010; 115(3): 167-174 .

57. Brumpt C, Joyeux. Description d'un nouvel echinocoque: Echinococcus cruzi n. sp. Ann Parasitol Hum Comp 1924 ; 3: 226-231 .

58. Cameron M, Thomas W. Observation on the genus Echinococcus Rudolphi. J Helminthol 1926; 1801 IV: 13-22.

59. Diesing K.M. Revision der Cephalocotyteen. Sitzungsb. K. Akad. Wissensch. Wien Math-Naturw. Abteilung: Paramecocotyleen. 1863, 48.

60. Ortlepp JR. Echinococcus in dogs from Pretoria and vicinity. Onderstepoor J Vet Sci Anim Ind 1934; 3: 97-108.

61. Ortlepp JR. South African Helminths-Part I. Onderstepoort J Vet Sci Anim Ind 1937; 9: 311-336.

62. Vogel H. Über den Entwicklungsgang und die Artzugehörigkeit des Europäischen Alveolar Echinococcus. Dtsch Med Wochenschr 1955; 80: 931-932.

63. Vogel H. Über den Echinococcus multilocularis Süddeutschlands. I. Das Bandwurmstadium von Stämmen menschlicher und tierischer Herkunft. Z Tropenmed Parasitol 1957; 8: 404-454.

64. Lopez-Neyra, Soler Planas. Revision del genero Echinococcus Rud y description de una especie nuéva Parasita intestinal del porro en Almeria. Rev IberParasitol 1943; 3: 169-194.

65. Szidat L. Echinococcus patagonicus sp. nov. (Cestoda) parasito del zorro Dusicyon culpaeus culpaeus (Mol.). Neotropica 1960; 6: 13-16.

66. Schantz U, Colli PM, Cruz-Reyes C, Prezioso A. Sylvatic echinococcosis in Argentina. 2. Susceptibility of wild carnivores to Echinococcus granulosus (Batsch 1786) and hostinduced morphological variation. Tropenmed Parasitol 1976; 27: 70-78.

67. Szidat L. Echinococcus cepanzoi sp. nov. (Cestoda Taeniidae) del zorro gris, Dusicyon gymnocercus, de la provincia de Buenos Aires. Neotropica 1971; 17: 1-4.

68. Szidat L. Echinococcus pampeanus—Una nueva specie de la Argentina, parasito de Felis colocolo pajeros Desmarest 1916 (Cestoda). Neotropica 1967 ; 13: 90-96 .

69. Rausch J J, Bernstein R L. Echinococcus vogeli spp. n. (Cestoda: Taeniidae) from the bush dog, Speothos venaticus (Lund.). Z Tropenmed Parasite 1972 ; 23: 25-34 .

70. Rausch EL, Schiller RL. Studies on the helminth fauna of Alaska XXV. Echinococcus sibiricensis n. sp. from St. Lawrence Island. J Parasitol 1954; 46(3-4): 659-662.

71. Shul'ts R S. On the epidemiology of echinococcosis and methods of its control. Med Parasite 1962 ; 31: 272.

72. Verster A J. Review of Echinococcus species in South Africa. Onderstepoort J Vet Res 1965; 32: 7-118.

73. Rausch R L. A consideration of infraspecific categories in the genus Echinococcus Rudolphi, 1801 (Cestoda: Taeniidae). J Parasitol 1967; 53: 484-491.

74. Kumaratilake LM, Thompson RC. A review of the taxonomy and speciation of the genus Echinococcus Rudolphi 1801. Z Parasitenkd 1982; 68: 121-146.

75. Bowles D P, Blair J, McManus D. Genetic variants within the genus Echinococcus identified by mitochondrial DNA sequencing. Mol Biochem Parasitol. 1992; 54(2): 165-173.

76. Bowles DP, McManus J. Molecular variation in Echinococcus. Acta Trop 1993; 53(3-4): 291-305.

77. Bowles D P, Blair J, McManus D. Molecular genetic characterization of the cervid strain ('northern form') of *Echinococcus granulosus*. Parasitol 1994 ; 109 (Pt 2): 215-221.

78. Lavikainen S, Lehtinen A, Meri MJ, Hirvela-Koski T, Meri V. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. Parasitol 2003; 127(Pt 3): 207-215.

79. Scott JC, Stefaniak J, Pawlowski ZS, McManus DP. Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotypic group (G9) of *Echinococcus granulosus*. Parasitol 1997; 114 (Pt 1): 37-43.

80. Xiao N, Qiu J, Nakao M et al. Echinococcus shiquicus, a new species from the Qinghai-Tibet plateau region of China: discovery and epidemiological implications. Parasitol Int 2006 ; 55 (Suppl): S233-S236.

81. Thompson RC, Lymbery AJ, Constantine CC. Variation in Echinococcus: towards a taxonomic revision of the genus. Adv. Parasitol 1995 ; 35: 145-176.

82. Thompson RC, McManus DP. Towards a taxonomic revision of the genus Echinococcus, Trends Parasitol 2002; 18(10): 452-457.

83. Webster GA, Cameron TWM. Observations on experimental infections with Echinococcus in rodents. Can J Zool 1961; 39: 877-889.

84. Nakao M1, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus Echinococcus inferred from complete mitochondrial genomes. Parasitol 2007; 134(Pt 5): 713-722.

85. Bart JM, Bardonnet K, Elfegoun MC et al. Echinococcus granulosus strain typing in North Africa: comparison of eight nuclear and mitochondrial DNA fragments. Parasitol 2004; 128: 229-234.

86. Zhang L, Gasser RB, Zhu X, McManus DP. Screening for different genotypes of Echinococcus granulosus within China and Argentina by single-strand conformation polymorphism (SSCP) analysis. Trans R Soc Trop Med Hyg 1999; 93: 329-334 .

87. Zhang LH, Chai JJ, Jiao W, Osman Y, McManus DP. Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of Echinococcus granulosus in north-western China. Parasitol 1998 ; 116 (Pt 1): 29-33.

88. Dinkel A, Njoroge EM, Zimmermann A et al. A PCR system for detection of species and genotypes of the Echinococcus granulosus-complex, with reference to the epidemiological situation in eastern Africa. Int J Parasitol 2004; 34(5): 645-53.

89. Busi M, Snábel V, Varcasia A et al. Genetic variation within and between G1 and G3 genotypes of *Echinococcus granulosus* in Italy revealed by multilocus DNA sequencing. Vet Parasitol 2007; 150(1-2): 75-83.

90. Casulli A, Interisano M, Sreter T,et al. Genetic variability of Echinococcus granulosus sensu stricto in Europe inferred by mitochondrial DNA sequences. Infect Genet Evol 2012; 12(2): 377-383 .

91. Snábel V, Altintas N, D'Amelio S et al. Cystic echinococcosis in Turkey: genetic variability and first record of the pig strain (G7) in the country. Parasitol Res 2009;105(1): 145-54.

92. Vural G, Baca AU, Gauci CG et al. Variability in the Echinococcus granulosus cytochrome C oxidase 1 mitochondrial gene sequence from livestock in Turkey and a re-appraisal of the G1-3 genotype cluster. Vet Parasitol 2008; 154(3-4): 347-50.

93. Yanagida T, Mohammadzadeh T, Kamhawi S,et al. Genetic polymorphisms of *Echinococcus granulosus sensu stricto* in the Middle East. Parasitol Int 2012 ; 61(4): 599-603.

94. Cardona GA., Carmena D. A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. Vet Parasitol 2013; 192(1-3): 10-32.

95. McManus DP, Thompson RC. Molecular epidemiology of cystic echinococcosis. Parasitol 2003; 127 (Suppl): S37-S51.

96. Alvarez Rojas CA, Romig T, Lightowlers MW. Echinococcus granulosus sensu lato genotypes infecting humans - review of current knowledge. Int J Parasitol 2014; 44(1) : 9-18.

97. Khalifa NO, Khater H, Fahmy HA, Radwan MEI. Genotyping and phylogenetic analysis of cystic echinococcosis isolated from camels and humans in Egypt. Am J Epidemiol Inf Dis 2014; V2: 74-82 .

98. Omer RA, Daugschies A, Romig T. Cystic echinococcosis in Sudan and South Sudan: research history of a neglected zoonosis. Berl Munch Tierarztl Wochenschr 2011; 124(11-12): 521-527.

99. Kumaratilake LM, Thompson RC, Eckert J. *Echinococcus granulosus* of equine origin from different countries possess uniform morphological characteristics. Int J Parasitol 1986; 16(5): 529-540.

100. Smyth J D. Strain differences in Echinococcus granulosus, with special reference to the status of equine hydatidosis in the United Kingdom. Trans R Soc Trop Med Hyg 1977; 71(2): 93-100 .

101. Bowles J, Knapen F, McManus DP. Cattle strain of *Echinococcus granulosus* and human infection. Lancet 1992; 339(8805): 1358.

102. LE T H, Pearson MS, Blair D, DAI N. Complete mitochondrial genomes confirm the distinctiveness of the horse–dog and sheep–dog strains of Echinococcus granulosus. Parasitol 2002; 124(Pt 1): 97-112.

103. Mitrea IL, Lonita M, Dumitrache IIC, Ciopasiu R. Prevalence and morphological characterisation of Echinococcus granulosus larvae in some livestock from the South areas of Romania. Sci Work C Ser Vet Med 2010; 56: 114-123.

104. Varcasia A, Garippa G, Pipia AP et al. Cystic echinococcosis in equids in Italy. Parasitol. Res 2008; 102(4): 815-818 .

105. Simsek S, Cevik A. First Detection and Molecular Characterization of Echinococcus equinus in a Mule in Turkey.Acta Parasitol 2014; 59(4): 773-777.

106. Simsek S, Roinioti E, Eroksuz H. First Report of Echinococcus equinus in a Donkey in Turkey. Korean J Parasitol 2015; 53(6): 731-735.

107. Thompson RC, Kumaratilake LM, Eckert J. Observations on Echinococcus granulosus of cattle origin in Switzerland. Int J Parasitol 1984 ; 14: 283-291.

108. Worbes H, Thompson RC, Eckert J. Occurrence of the cattle strain of Echinococcus granulosus in the German Democratic Republic. Parasitol Res 1989; 75(7): 495-497.

109. Hahn G, Kuske V, Hörchner F, Reuter G. Zur Verbreitung der zystischen Echinokokkose beim Rind in Deutschland. Dtsch. Tierarztl. Wochenschr 1986 ;93:445-447.

110. Omer RA, Dinkel A, Romig T et al. A molecular survey of cystic echinococcosis in Sudan. Vet Parasitol 2010; 169(3-4): 340-346.

111. Mbaya H, Magambo J, Njenga S et al. Echinococcus spp. in central Kenya: a different story. Parasitol Res 2014; 113(10): 3789-3794.

112. Mogoye BK, Menezes CN, Wong ML et al. First insights into species and genotypes of Echinococcus in South Africa. Vet Parasitol 2013; 196(3-4): 427-432.

113. Mário L. de La RueI, Anke Dinkel et al. New data on *Echinococcus spp*. in Southern Brazi. Rev Inst Med Trop Sao Paulo 2006; 48: 103-104.

114. Grenouillet F, Umhang G, Arbez-Gindre F et al. *Echinococcus ortleppi* infections in humans and cattle. France. Emerg Infect Dis 2014; 20(12): 2100-2.

115. Pednekar RP, Gatne ML, Thompson RC, Traub RJ. Molecular and morphological characterisation of Echinococcus from food producing animals in India. Vet Parasitol 2009; 165(1-2): 58-65.

116. Plesker R, Nadler T, Dinkel A, Romig T. A case of an Echinococcus ortleppi infestation in a red-shanked douc langur (Pygathrix nemaeus) in northern Vietnam. Vietnamese J Primatol. 2009; 3:75-81.

117. Boufana B, Stidworthy MF, Bell S et al. Echinococcus and Taenia spp. from captive mammals in the United Kingdom. Vet Parasitol 2012; 190(1-2): 95-103.

118. Cristian A, Rojas A, Romig T, Marshall W. *Echinococcus granulosus sensu lato* genotypes infecting humans – review of current knowledge. Int J Parasitol 2014; 44(1): 9-18.

119. Eckert J, Thompson RC, Lymbery AJ et al. Further evidence for the occurrence of a distinct strain of *Echinococcus granulosus* in European pigs. Parasitol Res 1993 ; 79: 42-48 .

120. Bowles J, Blair D, McManus DP. A molecular phylogeny of the genus Echinococcus, Parasitol 1995 ; 110 (Pt 3): 317-328 .

121. Lavikainen A, Lehtinen MJ, Laaksonen S et al. Molecular characterization of Echinococcus isolates of cervid origin from Finland and Sweden. Parasitol 2006; 133: 565-570.

122. Sweatman GK, Williams RJ. Comparative studies on the biology and morphology of Echinococcus granulosus from domestic livestock, moose and reindeer. Parasitol 1963; 53: 339-390 .

123. Thompson RC. The taxonomy, phylogeny and transmission of Echinococcus. Exp Parasitol 2008; 119(4): 439-446.

124. Sharma M, Sehgal R, Fomda BA et al. Molecular characterization of *Echinococcus granulosus* cysts in north Indian patients: identification of G1, G3, G5 and G6 genotypes. PLoS Negl Trop Dis 2013; 13;7(6): e2262.

125. Konyaev SV, Yanagida T, Nakao M et al. Genetic diversity of *Echinococcus spp*. in Russia, Parasitol 2013 ; 140(13): 1637-47.

126. Moks E, Jogisalu I, Valdmann H, Saarma U. First report of *Echinococcus granulosus* G8 in Eurasia and a reappraisal of the phylogenetic relationships of 'genotypes' G5-G10, Parasitol 2008; 135(5): 647-654.

127. Lymbery AJ, Jenkins EJ, Schurer JM, Thompson RC. *Echinococcus canadensis*, *E. borealis* and *E. intermedius*. What's in a name?. Trends Parasitol 2015; 31(1): 23-29.

128. Soriano SV, Pierangeli NB, Pianciola L et al. Molecular characterization of Echinococcus isolates indicates goats as reservoir for *Echinococcus canadensis* G6 genotype in Neuquén, Patagonia Argentina. Parasitol Int 2010; 59(4): 626-8.

129. Bart JM, Morariu S, Knapp J et al. Genetic typing of Echinococcus granulosus in Romania. Parasitol Res 2006; 98(2): 130-137.

130. Yang YR, McManus DP, Huang Y, Heath DD. *Echinococcus granulosus* infection and options for control of cystic echinococcosis in Tibetan communities of Western Sichuan Province, China. PLoS Negl Trop Dis 2009; 3(4) : e426.

131. Harandi MF, Hobbs RP, Adams PJ et al. Molecular and morphological characterization of *Echinococcus granulosus* of human and animal origin in Iran. Parasitol 2002; 125(Pt 4): 367-373.

132. Zhang LH, Joshi DD, McManus DP. Three genotypes of Echinococcus granulosus identified in Nepal using mitochondrial DNA markers. Trans R Soc Trop Med Hyg 2000; 94(3): 258-260.

133. Latif AA, Tanveer A, Maqbool A et al. Morphological andmolecular characterisationof Echinococcus granulosus inlivestock and humans in Punjab, Pakistan. Vet Parasitol 2010; 170(1-2): 44-49.

134. Hajialilo E, Harandi MF, Sharbatkhori M et al. Genetic characterization of Echinococcus granulosus in camels, cattle and sheep from the south-east of Iran indicates the presence of the G3 genotype. J Helminthol 2012; 86(3): 263-27.

135. Sobrino R, Gonzalez LM, Vicente J et al. Echinococcus granulosus (Cestoda, Taeniidae) in the Iberian wolf. Parasitol Res 2006;99(6): 753-756.

136. Eckert J, Thompson RC. Echinococcus strains in Europe: a review. Trop Med Parasitol 1988; 39(1): 1-8.

137. Blutke A, Hamel D, Huttner M et al. Cystic echinococcosis due to *Echinococcus equinus* in a horse from southern Germany. J Vet Diagn Invest 2010; 22(3):458-462.

138. Pawlowski Z, Stefaniak J. The pig strain of Echinococcus granulosus in humans: a neglected issue. Trends Parasitol 2003; 19(10): 439.

139. Schneider R, Gollackner B, Schindl M et al. *Echinococcus canadensis* G7 (pig strain): an underestimated cause of cystic echinococcosis in Austria. Am J Trop Med Hyg 2010; 82: 871-874.

140. Varcasia A, Canu S, Kogkos A et al. Molecular characterization of *Echinococcus granulosus* in sheep and goats of Peloponnesus, Greece. Parasitol Res 2007; 101(4): 1135-1139.

141. Mwambete DK, Ponce-Gordo F, Cuesta-Bandera C. Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. Acta Trop 2004; 91(2): 87–93.

142. Lukmanova GI, Gumeov AA, Tuˇıgunov MM, Viktorova TV. Genomic typing of the *Echinococcus granulosus* isolates from the areas of Southern Urals. Parazitologiia 2006; 40: 479-484.

143. Nikulina NA, Benediktov II, Garaev MM. *Echinococcus granulosus* genotyping by PCR-RFLP. Med Parazitol 2003; 2: 29-32.

144. Casulli A, Manfredi MT, La Rosa G et al. *Echinococcus ortleppi* and *E. granulosus* G1, G2 and G3 genotypes in Italian bovines. Vet Parasitol 2008; 155(1-2): 168-172.

145. Rinaldi L, Maurelli MP, Veneziano V et al. The role of cattle in the epidemiology of *Echinococcus granulosus* in an endemic area of southern Italy. Parasitol Res 2008; 103(1): 175-179.

146. Capuano F, Rinaldi L, Maurelli MP et al. Cystic echinococcosis in water buffaloes: epidemiological survey and molecular evidence of ovine (G1) and buffalo (G3) strains. Vet Parasitol 2006; 137(3-4): 262-8.

147. Beyhan YE, Umur S. Molecular characterization and prevalence of cystic echinococcosis in slaughtered water buffaloes in Turkey. Vet Parasitol 2011; 181(2-4): 174-179.

148. Blutke A, Hamel D, Huttner M, et al. Cystic echinococcosis due to *Echinococcus equinus* in a horse from southern Germany. J Vet Diagn Invest 2010; 22(3): 458-462.

149. Romig T, Dinkel A, Mackenstedt U. The present situation of echinococcosis in Europe. Parasitol Int 2006; 55 (Suppl): S187-S191.

150. Bruzinskaite R, Sarkunas M, Torgerson PR, Mathis A, Deplazes P. Echinococcosis in pigs and intestinal infection with *Echinococcus spp*. in dogs in southwestern Lithuania. Vet Parasitol 2009; 160(3-4): 237-241.

151. Bart JM, Abdukader M, Zhang YL, et al. Genotyping of human cystic echinococcosis in Xinjiang, PR China. Parasitol 2006; 133(Pt 5): 571-579.

152. Hüttner M, Siefert L, Mackenstedt U, Romig T. A survey of Echinococcus species in wild carnivores and livestock in East Africa. Int J Parasitol 2009; 39(11): 1269-1276.

153. Obwaller A, Schneider R, Walochnik J et al. *Echinococcus granulosus* strain differentiation based on sequence heterogeneity in mitochondrial genes of cytochrome c oxidase-1 and NADH dehydrogenase-1. Parasitol 2004; 128: 569-575.

154. Schneider R, Gollackner B, Edel B et al. Development of a new PCR protocol for the detection of species and genotypes (strains) of Echinococcus in formalin-fixed, paraffin-embedded tissues. Int J Parasitol 2008; 38: 1065-1071.

155. Utuk AE, Simsek S, Koroglu E, McManus DP. Molecular genetic characterization of different isolates of *Echinococcus granulosus* in east and southeast regions of Turkey. Acta Trop 2008; 107: 192-194.

156. Simsek S, Eroksuz Y. Occurrence and molecular characterization of *Echinococcus granulosus* in Turkish mouflon (Ovis gmelinii anatolica). Acta Trop 2009; 109(2): 167-169.

157. Simsek S, Balkaya I, Koroglu E. Epidemiological survey and molecular characterization of *Echinococcus granulosus* in cattle in an endemic area of eastern Turkey. Vet Parasitol 2010 ; 172: 347-349.

158. Ergin S, Saribas S, Yuksel P et al. Genotypic characterisation of *Echinococcus granulosus* isolated from human in Turkey. Afr J Microbiol Res 2010; 4: 551-555.

159. Simsek S, Kaplan M, Ozercan IH. A comprehensive molecular survey of *Echinococcus granulosus* in formalin-fixed paraffin-embedded tissues in human isolates in Turkey. Parasitol Res 2011; 109(2): 411-6.

160. Kesik H K, Simsek S, Gunyakti S, Kilincab, Koroglu E. Identification of antigen B (AgB) Gene polymorphism in cattle and sheep isolates of *Echinococcus granulosus* and investigation of effects on serological diagnosis. Acta Tropica 2019; 199.

161. Torgerson PR, Macpherson CN.The socioeconomic burden of parasitic zoonoses: global trends. Vet Parasitol 2011; 182 (1): 79-95.

162. Sadjjadi SM. Present situation of echinococcosis in the Middle East and Arabic North Africa. Parasitol Int 2006; 55: S197-202.

163. Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus Echinococcus inferred from complete mitochondrial genomes. Parasitol. 2006; 134: 713-22.

164. 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.

165. Al-Kitani F, Baqir S, Hussain MH, Roberts D. Cystic hydatidosis in slaughtered goats from various municipal abattoirs in Oman. Trop Anim Health Prod 2014; 46(8): 1357-62.

166. Al-Kitani F, Baqir S, Hussain MH, Robert D. Prevalence and Molecular Characterization of *Echinococcos granulosus* in Cattle in Oman. Global Veterinaria 2015; 14 (6): 883-891.

167. Kebede W, Hagos A, Girna Z, Lobago F. Echinococcosis/hydatidosis:Its prevalence, economic and public health significance in Tigray region. Tropical Animal Health and Production 2009; 41: 865-871.

168. Sayek I, Tirnaksiz MB, Dogan. Cystic hydatid disease: current trends in diagnosis and management. R Surg Today 2004; 34(12): 987-96.

169. Eryildiz C, Şakru, N. Molecular characterization of human and animal isolates of *Echinococcus granulosus* in the Thrace region, Turkey. Balkan Medical Journal. 2012; 29: 261-267.

170. Rostami S, Talebi S, Babaei Z et al. High resolution melting technique for molecular epidemiological studies of cystic echinococcosis: differentiating G1, G3, and G6 genotypes of *Echinococcus granulosus sensu lato*. Parasitol Res 2013; 112: 3441-3447.

171. Smyth JD, Smyth MM. Natural and experimental hosts of *Echinococcus granulosus* and *E. multilocularis*, with comments on the genetics of speciation in the genus Echinococcus. Parasitol 1964; 54: 493-514.

172. Casulli A, Interisano M, Sreter T et al. Genetic variability of *Echinococcus granulosus sensu stricto* in Europe inferred by mitochondrial DNA sequences. Infection Genetics Evol 2012; 12: 377-383.

173. Kinkar L, Laurimäe T, Acosta-Jamett G et al. Global phylogeography and genetic diversity of the zoonotic tapeworm *Echinococcus granulosus sensu stricto* genotype G1. Int J Parasitol 2018; 48(9-10): 729-742.

174. Sharma M, Fomda B.A, Mazta S et al. Genetic diversity and population genetic structure analysis of *Echinococcus granulosus sensu stricto* complex based on mitochondrial DNA signature. PLoS One 2013 ; 8: e82904.

175. Alvarez Rojas CA, Ebi D, Gauci CG et al. Microdiversity of *Echinococcus granulosus sensu stricto* in Australia. Parasitol 2016; 143: 1026-1033.

176. Debeljak Z, Boufana B, Interisano M et al. First insights into the genetic diversity of *Echinococcus granulosus sensu stricto (s.s.)* in Serbia. Vet Parasitol 2016; 223: 57-62.

177. Hassan ZI, Meerkhan AA, Boufana B et al. Two haplotype clusters of *Echinococcus granulosus sensu stricto* in northern Iraq (Kurdistan region) support the hypothesis of a parasite cradle in the Middle East. Acta Trop 2017; 172: 201-207.

178. Kamenetzky L, Gutierrez AM, Canova SG et al. Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. Infect Genet Evol 2002 ; 2: 129-136.

179. Haag KL, Ayala FJ, Kamenetzky L et al. Livestock trade history, geography, and parasite strains: the mitochondrial genetic structure of *Echinococcus granulosus* in Argentina. J Parasitol. 2004 ; 90: 234-239.

180. Badaraco JL, Ayala FJ, Bart JM et al. Using mitochondrial and nuclear markers to evaluate the degree of genetic cohesion among Echinococcus populations. Exp Parasitol 2008; 119: 453-459.

181. Nakao M, Li M, Han X et al. Genetic polymorphisms of Echinococcus tapeworms in China as determined by mitochondrial and nuclear DNA sequences. Int J Parasitol 2010; 40(3): 379-385.

182. Knapp J, Gottstein B, Saarma U, Millon L.Taxonomy, phylogeny and molecular epidemiology of *Echinococcus multilocularis*: from fundamental knowledge to health ecology. Vet Parasitol 2015; 213: 85-91.

183. Laurimaa L, Davison J, Süld K et al. First report of highly pathogenic *Echinococcus granulosus* genotype G1 in dogs in a European urban environment. Parasites Vectors 2015; 8: 182.

184. Alvarez Rojas CA, Romig T, Lightowlers MW. *Echinococcus granulosus sensu lato* genotypes infecting humans - review of current knowledge. Int J Parasitol. 2014; 44: 9-18.

185. al Karawi MA, el-Shiekh Mohamed AR, Yasawy MI. Advances in diagnosis and management of hydatid disease. Hepatogastroenterology 1990; 37: 327-31.

186. Ammann R. Cestodes: Echinococcus. Gastroenterol Clin N Am 1996; 25: 655-89.

187. Yong W, Heath D. Comparison of cestode antigens in an enzyme-linked immunosorbent assay for the diagnosis of *Echinococcus granulosus, Taenia hydatigena* and *Taenia ovis* infections in sheep. Res Vet Sci 1984; 36(1): 24-31.

188. Akbulut S, Senol A, Sezgin A, Cakabay B. Radical vs conservative surgery for hydatid liver cysts: experience from single center. World J Gastroenterol 2010; 16(8): 953-9 .

189. Aydin U, Yazici P, Onen Z, Ozsoy M and C. A. Kiliç M. The optimal treatment of hydatid cyst of the liver: radical surgery with a significant reduced risk of recurrence. Turk J Gastroenterol 2008; 19(1): 33-9.

190. Motie MR, Ghaemi M, Aliakbarian M. Study of the radical vs. conservative surgical treatment of the hepatic hydatid cyst: a 10- year experience. Indian J Surg 2010; 72(6): 448-52 .

191. Secchi MA, Pettinari R, Mercapide C et al. Surgical management of liver hydatidosis: a multicentre series of 1412 patients. Liver Int 2009; 30: 85–93 .

192. Wen H, Shao YM, Zhao JM, Tuergan A. Clinical analysis of surgical treatment for human hepatic cystic and alveolar echinococcosis. Chinese J Dig Surg 2007; 6: 13-8 .

193. Conchedda M, Ecca A, Gabrielle F, Bortoletti G. Cestode Zoonoses: Echinococcosis and Cysticercosis: An Emergent and Global Problem. Emerg Infect Dis 2002; 8(11); 1362.

194. Gemmell M, Roberts M, Beard T, Campano-Diaz S. Control of echinococcosis, a public health problem of global concern. M.A. Gemmell, F.-X. Meslin and Z.S. Pawłowski J. Eckert. WHO/OIE Manual on Echinococcosis in Humans and Animals:a Public Health Problem of Global Concern . 2001. 195-204.

195. Woollard DJ1, Heath DD, Lightowlers MW. Assessment of protective immune responses against hydatid disease in sheep by immunization with synthetic peptide antigens. Parasitol 2000 ;121 (Pt2): 145-153.

196. Beiromvand M, Akhlaghi L, Fattahi Massom SH, et al. Detection of *Echinococcus multilocularis* in carnivores in Razavi Khorasan province, Iran using mitochondrial DNA. PLoS Negl Trop Dis 2011; 5(11): e1379.

197. Breyer I, Georgieva D, Kurdova R, Gottstein B. *Echinococcus granulosus* strain typing in Bulgaria: the G1 genotype is predominant in intermediate and definitive wild hosts. Parasitol Res 2004; 93(2): 127-30.

198. Guerra D, Armua-Fernandez MT, Silva M et al. Taeniid species of the Iberian wolf (Canis lupus signatus) in Portugal with special focus on *Echinococcus spp*. Int J Parasitol Parasites Wild 2012; 2: 50-3.

199. Schurer J, Shury T, Leighton F, Jenkins E. Surveillance for *Echinococcus canadensis* genotypes in Canadian ungulates. Int J Parasitol 2013; 2: 97-101.