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Short communication

Multiple Coenurus cerebralis Cysts Detected in a Sheep Brain and Molecular Characterization of the Individual Cysts

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Abstract

Taenia multiceps is found in canids and in its larval stage is known as *Coenurus cerebralis* causes coenurosis. The disease has a significant impact on the economic value of sheep and goats. The aim of the current study was to identify multiple cysts in the brain of a sheep displaying common symptoms of *C. cerebralis* and to amplify and sequence analyse the mitochondrial cytochrome oxidase subunit 1 gene of each individual cyst by PCR. The research material used was the head of a sheep exhibiting neurological symptoms. Seven cysts associated with *C. cerebralis* were detected in the brain upon thorough examination. The mt-CO1 gene was amplified by PCR, and all isolates were sequenced. Sequence alignment revealed the presence of point mutations, and 20 polymorphic sites were identified, of which 7.7% (1/13) were parsimony informative. The isolates demonstrated significant haplotype diversity and low nucleotide diversity. In this study, only one isolate obtained from Turkey belonged to the fourth main haplotype, while the remaining six isolates constituted a distinct and unique single haplotype. This is the first time that haplotypic distinctions have been identified among isolates obtained from a sheep brain that is multiply infected with *C. cerebralis*.

Keywords

Coenurus cerebralis, haplotype, mt-CO1, PCR, sheep, Taenia multiceps

Introduction

Coenurosis is a parasitic disease caused by the larva of *Taenia multiceps* which can result in significant economic losses for sheep and goats (Abera et al., 2016). Although information about the extent of damage caused by coenurosis is limited, it is responsible for flock infections in sheep and can cause significant harm (Scala et al., 2007). During its larval stage, *C. cerebralis* forms fluid-filled cysts that measure between 0.8 and 6.5 cm in diameter when intermediate hosts settle in the central nervous system (CNS). Infected animals exhibit neurological symptoms, such as turning, ataxia, head deviation, and blindness (Abera et al., 2016). Recent studies suggest that clinical and pathological manifestations are not only related to the enlarged cyst volume but also to neurotoxicity (Liu et al., 2019). Coenurosis is a common condition worldwide. Farmers often choose to slaughter animals as soon as they notice the first symptoms of coenurosis to maintain carcass yield and prevent losses resulting from the progression of the illness. This practice explains the relatively low incidence of coenurosis (Scala et al., 2007).

The cyst count in infected sheep typically ranges from 1-4 cysts per animal in most studies (Anwar et al., 2013; Gholami et al., 2022; Rahsan et al., 2018; Uslu and Guclu, 2007). However, Achenef et al. (1999) observed a range of 1-8 cysts per animal. It is worth noting that Amer et al. (2017) reported an interesting discovery, identifying 40 small cysts in the brain of a sheep exhibiting typical symptoms of *C. cerebralis* in Egypt.

This study was planned to identify multiple cysts detected in the brain of a sheep showing typical signs of *C. cerebralis* and to perform sequence and haplotype analyses of each cyst.

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Materials and methods

A 1-year-old Akkaraman sheep was examined by a veterinarian before slaughter in a private slaughterhouse located in Elazig province of Türkiye. The sheep showed signs of dullness, reluctance to move, drooling, dizziness, frequent convulsions, ataxia and decreased appetite. According to the sheep owner, no other sheep in the flock showed symptoms as typical as this sheep and he decided to slaughter the sheep to prevent further weight loss. After slaughter, the sheep's head was transported to the laboratory due to clinical signs resembling coenurosis. In the laboratory, the parietal bone of the sheep was cut through two parallel incisions just caudal to the frontal bone and the bone was removed with a chisel and hammer. The meninges were cut with a scalpel blade to expose the brain. Individual cyst specimens were removed and placed in Petri dishes. After discarding the cyst fluid, the germinal membrane and protoscoleces were mixed with 70% ethanol and stored at -20°C until further analysis.

The genomic DNA was isolated from each individual cyst sample separately using the DiaRex Tissue Kit (Diagen, Türkiye) with minor modifications. A surgical blade was used to crush the protoscoleces, and the resulting tissue samples were transferred to 1.5 mL Eppendorf tubes. The samples were washed at least five times with PBS (pH=7.4) to remove ethanol residues. Then, 300 µL of lysis solution was added to the tubes and mixed thoroughly until homogenous. 25 µL of Proteinase-K (20 mg/ml, Hibrigen, Türkiye) was added to the samples and mixed using a vortex. The mixture was then incubated in a water bath at 56°C overnight to allow for protoscoleces digestion. To ensure complete digestion, the samples were vortexed at least 2-3 times at one-hour intervals. The following day, genomic DNA was isolated following kit procedures and stored at -20°C until use.

The mt-CO1 gene fragment, consisting of 875 base pairs, was amplified using the primer set 5'-TTGAATTTGCCACGTTTGAATGC-3' and 5'-GAACCTAACGACATAACATAATGA-3' (Nakao et al., 2000). The amplification process involved an initial denaturation at 94 °C for 10 minutes, followed by 30 cycles at 94 °C for 30 seconds, 52 °C for 45 seconds, and 72 °C for 1 minute, and a final extension step at 72 °C for 10 minutes. The PCR reactions were preceded by a negative control using distilled water. Following electrophoresis in a 1.4% agarose gel, the PCR products were purified and performed one-way sequence analyses using the forward primer (F/CO1) (BM Labosis, Türkiye). The resulting sequences were compared with those in the GenBank reference sequences using a BLAST search tool (http://www.ncbi.nlm.nih.gov/BLAST/). After trimming the sequence ends, all sequences were uploaded to MEGA X (Kumar et al., 2018). DNA sequences were aligned using published reference sequences, with additional NCBI sequences added as outgroups. The most accurate evolutionary tree model was determined using the maximum likelihood method in MEGA X (Kumar et al., 2018).

The haplotype analysis was conducted using a published protocol, and the sequence data were uploaded to DnaSP6 software for analysis. Population diversity and neutrality indices were computed using DnaSP 6 (Rozas et al., 2017). NEXUS output formats were generated using the same software. A haplotype network that includes all edges found in a minimum spanning tree was created using the minimum spanning networks method and PopART 1.7 (Leigh and Bryant, 2015).

Results

The brain exhibited multiple cysts caused by *C. cerebralis* (Figure 1). Upon examination, seven distinct cysts of *C. cerebralis* were found throughout all regions of the brain. These cysts consisted of a hyaline membrane with numerous protoscoleces on its inner surface containing translucent fluid of varying volumes. An 875 bp fragment of the mt-CO1 gene was successfully amplified by PCR in all seven samples. The nucleotide sequences were compared with published reference sequences and trimmed accordingly to a final size of 818 base pairs for seven sequences (TmEL1-TmEL7). Based on BLAST searches, all seven sequences were identified as *T. multiceps*. The sequences were then submitted to NCBI and published under the following accession numbers: OR782913-OR782919.

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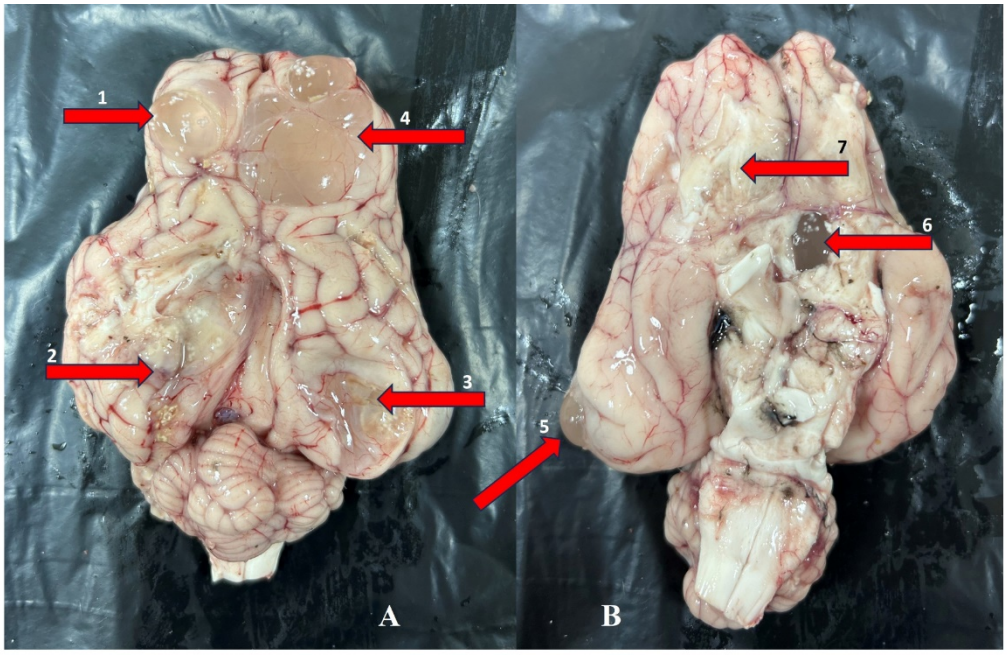

Figure 1. Appearance of cysts detected in the brain of a sheep naturally infected with Coenurus cerebralis. A: Dorsal view B: Ventral view.

After sequence alignment, point mutations were identified. Table I presents the positions of nucleotide variation based on the reference sequence (NC_012894). An 818 bp long fragment of the mt-CO1 illustrates a phylogenetic tree representing *C. cerebralis* samples (n = 7) detected in the brain of the sheep. Phylogenetic tree construction utilized sequences from *T. multiceps* (FJ495086, JX535575, NC_012894, MK189452), *T. saginata* (NC_009938), *T. ovis* (NC_021138), *T. solium* (NC_004022), *T. hydatigena* (NC_012896), and *Echinococcus granulosus* (MN787525). The maximum likelihood tree was constructed using MEGA X and the TN93+G model (Figure 2).

Nucleotide Positions	39	75	141	157	159	193	322	342	354	390	402	432	435	450	462	470	534	576	588	615	678	682	694	700	714	719	723	728	734	753	755	764	768	788	800
NC_012894 Reference sequence	T	A	A	A	T	A	T	T	C	T	G	T	C	A	A	T	C	A	G	A	T	C	G	T	T	T	T	T	G	T	T	A	T	T	T
Hap01	C								T	A	A						T			T					C		C		C	A	C	G	C	A	
Hap02	C								T	A	A						T																		
Hap03	C								T	A	A						T					T										G	C		
Hap04	C								T	A	A						T															G	C		C
Hap05	C								T	A	A						T						A	G								G	C		
Hap06	C								T	A	A						T															G	C		
Hap07	C								T	A	A						T																C		G
Hap08																																			
Hap09	C				C				T	A	A						T																		
Hap10			G							C	A		T				T	G			C														
Hap11			G					C		C	A		T				T	G			C														
Hap12			G			G				C	A		T				T	G	A		C														
Hap13										C	A		T		G		T	G			C	G													
Hap14		G					C			C	A		T	G	G		T	G			C	G													
Hap15											A		T				T	G			C														
Hap16			G							C	A	C	T				T	G			C														
Hap17										C	A		T		G	C	T	G			C	G				C									
Hap18										C	A		T				T	G			C														
Hap19										C	A		T				T	G																	
Hap20				G																															
Hap21																												C							

Table I. Nucleotide variation positions of the mt-CO1 (818 bp) gene among 21 analyzed haplotypes. A: Adenine, T: Thymine, C: Cytosine, G: Guanine.

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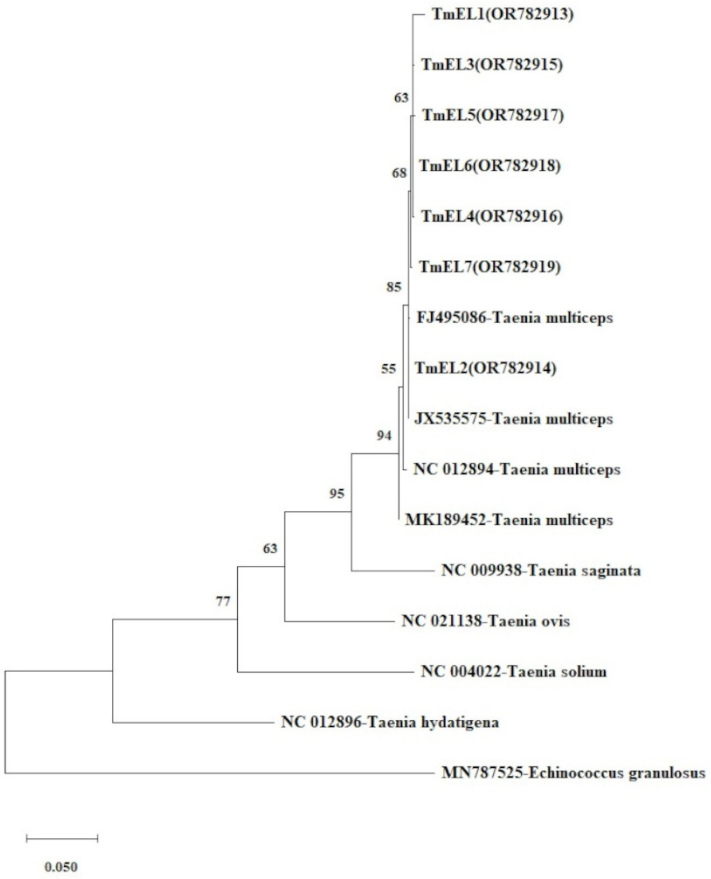

Figure 2. . A phylogenetic tree was constructed for Coenurus cerebralis samples (n = 7) detected in the brain of a sheep. The tree was based on an 818 bp long fragment of the mt-CO1 gene. Outgroup sequences including Taenia multiceps (FJ495086, JX535575, NC_01289, MK189452), T. saginata (NC_009938), T. ovis (NC_021138), T. solium (NC_004022), T. hydatigena (NC_012896), and Echinococcus granulosus (MN787525) were used for the construction of the phylogenetic tree. The Maximum Likelihood method was used to construct the tree based on the TN93+G model. The reliability of the tree was assessed by 1,000 bootstrap replications.

In this study, we identified twenty polymorphic sites among the *C. cerebralis* isolates. Of these, only 7.7% (1/13) were parsimony informative. The isolates showed high haplotype diversity but low nucleotide diversity, as shown in Table 2. The negative value of Tajima's D in the sequences indicates population expansion and/or purifying selection. Fu's F values were calculated from the sequences, indicating the emergence of uncommon haplotypes that may have resulted from a recent population expansion or hitchhiking. The presence of distinct singleton haplotypes in the mt-CO1 sequences of *C. cerebralis* is consistent with the configuration of the haplotype networks for the isolates (Figure 3).

mt-DNA	n	H	hd ± SD	πd ± SD	Tajima's D	P value	Fu's F	P value	FLD	P value	FLF	P value
CO1	7	7	1,000±0,076	0,00506±0,00151	-1,51353	P > 0.05	-3,487	0,030	-1,51116	P > 0.10	-1,66222	P > 0.10

Table II. Diversity and neutrality indices obtained using nucleotide data of the Coenurus cerebralis mt-CO1 gene (818 bp). n: Number of isolates; H: Number of haplotypes; hd: Haplotype diversity; πd: Nucleotide diversity; SD: Standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F statistics test. Not significant, P > 0.05; Not significant, P > 0.10,

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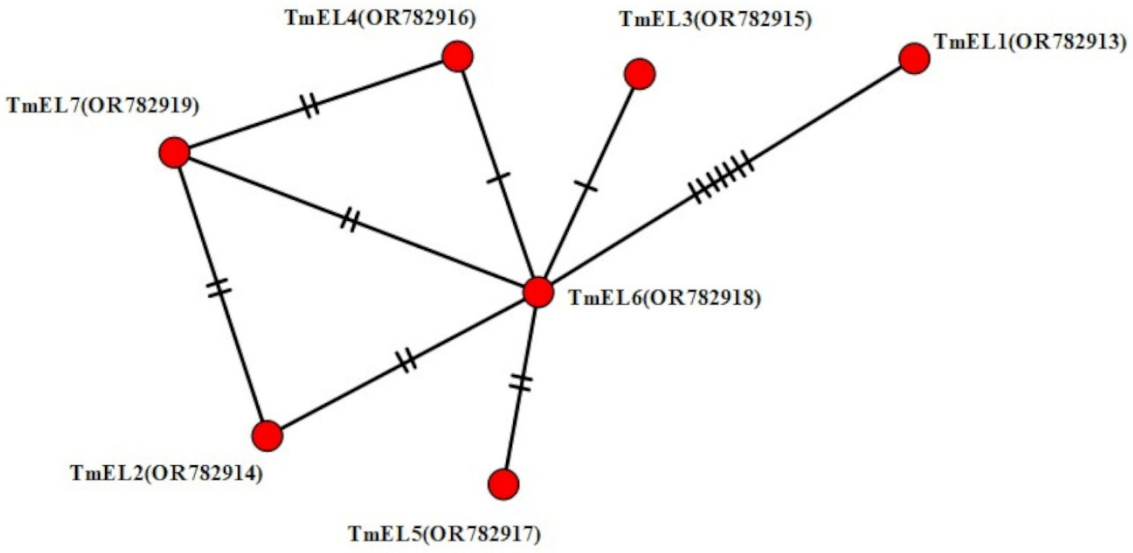

Figure 3. Frequencies of mt-CO1 (818 base pairs) haplotypes of C. cerebralis isolates from Türkiye. The size of each circle corresponds to the frequency of the haplotype. Hatch marks indicate additional mutational steps.

An analysis of haplotype was conducted on an 818 bp fragment of the mt-CO1 gene sequence from *T. multiceps* isolates. 35 variable points were found in the 42 sequences examined (Table 3). The mt-CO1 sequences showed an overall haplotype and nucleotide diversity of 0.933±0.020 and 0.00744±0.00066, respectively. The neutrality indices for the mt-CO1 gene were significantly negative (Tajima's D -1.08064, not statistically significant, $P > 0.01$; Fu's F -5.598, $P = 0.002$) (Table 4). Among the 42 isolates of *T. multiceps*, 21 haplotypes were identified in the mt-CO1 sequences (Figure 4). The haplotype network showed a star-like expansion, with the main haplotype (Hap10) occupying a central position and being separated from other haplotypes by one to 15 stepwise mutations. This haplotype was present in 7 (16.6%) of the total 42 isolates. Only seven goat isolates in China belonged to this main haplotype. In this study, only one of the isolates obtained from Türkiye belonged to the fourth main haplotype, while the remaining six isolates constituted a distinct and unique single haplotype.

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No	Haplotype name	Number of isolates	Isolate code (Accession number)-country-host
1	Hap01	1	TmEL1 (OR782913)-Turkey-Sheep
2	Hap02	4	TmEL2 (OR782914)-Turkey-Sheep JX535575-China-Sheep JX535570-China-Sheep JX535567-China-Sheep
3	Hap03	1	TmEL3(OR782915)-Turkey-Sheep
4	Hap04	1	TmEL4 (OR782916)-Turkey-Sheep
5	Hap05	1	TmEL5 (OR782917)-Turkey-Sheep
6	Hap06	1	TmEL6 (OR782918)-Turkey-Sheep
7	Hap07	1	TmEL7 (OR782919)-Turkey-Sheep
8	Hap08	5	NC_012894-China-Dog GQ228818-China-Dog CM010318-China-Dog JX535569-China-Sheep JX535574-China-Sheep
9	Hap09	1	FJ495086-China-Sheep
10	Hap10	7	JX507239-China-Goat JX507238-China-Goat JX507236-China-Goat JX507231-China-Goat JX507228-China-Goat JX507222-China-Goat JX507221-China-Goat
11	Hap11	1	JX507237-China-Goat
12	Hap12	1	JX507235-China-Goat
13	Hap13	6	JX507234-China-Goat JX507232-China-Goat JX507229-China-Goat JX507226-China-Goat JX507225-China-Goat JX507224-China-Goat
14	Hap14	1	JX507233-China-Goat
15	Hap15	1	JX507230-China-Goat
16	Hap16	1	JX507227-China-Goat
17	Hap17	1	JX507223-China-Goat
18	Hap18	1	JX507220-China-Goat
19	Hap19	4	JX535576-China-Sheep JX535572-China-Sheep JX535568-China-Sheep MK189452-China-Yak
20	Hap20	1	JX535573-China-Sheep
21	Hap21	1	JX535571-China-Sheep

Table III. Haplotypes of mt-CO1 sequences of Taenia multiceps and accession numbers of isolates forming groups.

mt-DNA	n	H	hd ± S.D	πd ± S.D	Tajima's D	P value	Fu's F	P value	FLD	P value	FLF	P value
CO1	42	21	0,933±0,020	0,00744±0,00066	-1,08064	P > 0.10	-5,598	0,002	-3,18908	*, P < 0.05	-2,82801	*, P < 0.05

Table IV. Diversity and neutrality indices obtained using nucleotide data of the Taenia multiceps mt-CO1 gene (818 bp). n: Number of isolates; hn: Number of haplotypes; hd: Haplotype diversity; πd: Nucleotide diversity; SD: Standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F statistics test. Not significant, P > 0.10, *, P < 0.05

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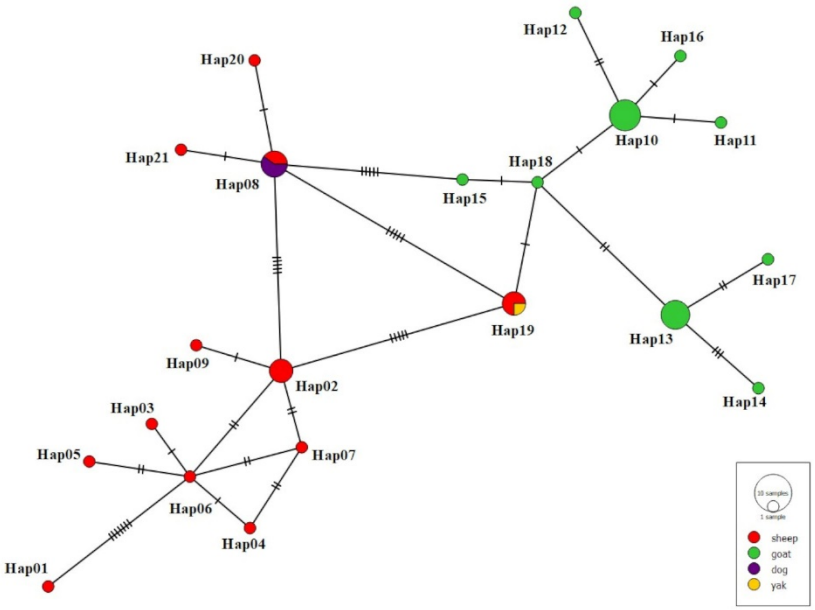

Figure 4. Haplotype network of Taenia multiceps isolates from various host using the mt-CO1 (818 bp) mitochondrial gene. Size of circles is proportional to the frequency of each haplotype. Number of mutations distinguishing the haplotypes is shown by hatch marks. Hap means haplotype.

Discussion

The issue of intraspecific variation in the life cycle and prevalence of taeniid cestodes is significant. To carry out effective protection and control programmes against these parasites, it is crucial to have a good understanding of the extent and nature of intraspecific genetic variation (Liu et al. 2011). The analysis of mt-CO1 and nad1 revealed three genetic variants of *T. multiceps*, namely Tm1, Tm2, and Tm3. Tm1 is the most commonly identified variant in Italy (Varcasia et al., 2006). Phylogenetic methods were used to analyse *T. multiceps* mt-CO1 gene sequences obtained from sheep isolates in Inner Mongolia and China. The analysis showed that there were three genotypes with variation rates ranging from 0.25% to 0.75% (Li et al., 2013). Zhang et al. (2018) was conducted a study to investigate the genetic diversity of *T. multiceps* in the Sichuan region of China and the levels of genetic variations detected 1.18%, 0.61% and 0.52% at the cox1, nad4 and cytb, respectively. Sonmez et al. (2017) identified three distinct variants in sheep from Türkiye using mt-CO1 gene sequences. The study revealed exceptionally low sequence variation. Point mutations were identified after aligning the current sequence results. The study also examined various polymorphic events in populations of *C. cerebralis* through four neutrality tests. Fu's Fs is particularly sensitive to past population growth, with large negative values typically indicating population expansion (Fu, 1997). Significant Tajima's D values suggest population expansion or bottleneck events (Tajima, 1989), while significant Fu and Li's D values indicate historical population shrinkage (Fu, 1997; Fu and Li, 1993).

Amer et al. (2017) demonstrated that *T. multiceps* isolates from sheep in Egypt exhibited low genetic diversity, with only a few haplotypes in the mt-CO1 and nad1 genes. In contrast, *T. multiceps* isolates obtained from sheep and goats in Iran showed significant genetic diversity, with seven haplotypes identified from 11 segregation sites in the mt-CO1 gene (Akbari et al., 2015; Rostami et al., 2013). Alvi et al. (2020) conducted a study in Pakistan which identified different *T. multiceps* haplotypes in sheep and goats. The haplotypes showed high haplotype diversity and low nucleotide diversity. The study employed a phylogenetic analysis to group the Pakistani and Chinese isolates into a cluster that was sufficiently distant from another cluster consisting of isolates from other countries. Varcasia et al. (2016) identified cases of polycystic coenurosis with multiple cysts in the central nervous system. These were found in 13 out of 47 animals, which accounts for 27.7% of the animals studied. However, previous studies were conducted on diverse isolates. The aim of current report was to identify the genetic variations in polycystic coenurosis (n=7) present in a sheep's brain. Only one of the isolates obtained was similar to previously recognised haplotypes, while the remaining six isolates formed a unique haplotype.

This study identified various haplotypes in different cysts of the brain for the first time. It is possible that individual

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cysts can arise from the eggs of different parasite individuals or through cross-fertilisation between adult parasites present in the intestine of the final host. In both cases, intraspecific variation in *T. multiceps* is rapidly spreading, resulting in the discovery of new haplotypes.

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