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Original Article

Differentiation of Cerebral Cystic Echinococcosis (CCE) from Coenurosis Using Morphometric and Molecular Methods

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Abstract

Background: Cerebral cystic echinococcosis (CCE) and coenurosis are zoonotic diseases caused by the larval stages of *Echinococcus granulosus sensu lato (s.l.)* and *Taenia* spp., respectively. Due to the similarity between the symptoms and clinical samples of CCE and cerebral coenurosis, especially in cases with no protoscoleces, the diagnostic methods for the differentiation of CCE from cerebral coenurosis are crucial, especially in countries where both diseases are endemic. To compare CCE and coenurosis, morphometric indices of protoscoleces and molecular methods were used in the present study.

***Methods*:** In this regard, four isolates of human cerebral echinococcal cysts, three isolates of *Coenurus cerebralis* from sheep, and one non-cerebral *Coenurus* from sheep muscles were evaluated. The isolated specimens have been collected from Shiraz, Ahvaz, Tehran and Kerman from before 2000 to 2022. The molecular characterization was carried out using the partial NADH dehydrogenase1 (nad1) gene. Phylogenetic analysis was performed using the maximum likelihood method.

***Results*:** In fertile cysts, the total size of the large and small hooks of *Coenurus* was larger than cerebral echinococcal cyst. These parameters demonstrated significant morphological differences between the *C. cerebralis* and the cerebral echinococcal cyst. Molecular methods identified the cerebral echinococcal cysts as *E. canadensis* (G6) genotype. One *C. cerebralis* and the non-cerebral *Coenurus* were identified as *Taenia multiceps* and *T. multiceps gaigeri*, respectively.

***Conclusion*:** Morphometric indices are significantly different between protoscoleces of *C. cerebralis* and cerebral echinococcal cysts. Hence, they could be used for differential diagnosis of the fertile cysts of these cestodes. However, in cases with no protoscoleces, molecular methods are essential for the differentiation of CCE from cerebral coenurosis, especially in regions where both diseases are prevalent and endemic.

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Introduction

Cystic echinococcosis (CE), caused by the larval stage of *Echinococcus granulosus sensu lato (s.l.)*, is one of the most important zoonotic diseases worldwide (1, 2). The definitive hosts of *E. granulosus s.l.* are canids, including dogs, wolves, foxes, and jackals, and the intermediate hosts are herbivores such as sheep, goats, cattle, and camel. Humans, as accidental intermediate host, become infected by ingestion of food or water contaminated with the eggs or through direct contact with an infected dog. CE develops almost anywhere in the human body, mainly in the liver and lungs. Brain involvement is rare, and it is more common in children than in adults (3). Cerebral cystic echinococcosis (CCE) in humans has been reported from different parts of the world, and its incidence is approximately 1-2% of all CE infections (4, 5).

E. granulosus s.l. contains 10 genotypes (G1 to G10) that are now recognized as separate species, including *E. granulosus sensu stricto* (G1-G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6- G8 and G10) (2, 6). Organ involvement depends on the genotype, for example, the G6 genotype has a higher affinity for the brain in humans (7, 8). Cerebral echinococcal cysts are classified as primary or secondary cysts. The primary cysts are formed as a result of direct infection of the larvae in the brain without demonstrable involvement of other organs, while the secondary cysts result from spontaneous, traumatic, or surgical rupture of the primary echinococcal cysts (9). Cerebral echinococcal cysts are found anywhere in the brain and are usually located in the middle cerebral artery (10). The definitive diagnosis of CCE is based on imaging methods. Computerized tomography (CT) and magnetic resonance imaging (MRI) are the most commonly used for diagnosis. The CCE may be misdiagnosed with brain tumors, neurocysticercosis, or cerebral coenurosis (11).

Coenurosis is a rare zoonotic disease caused by *Coenurus*, the larval stage of different *Taenia* species, including *T. multiceps*, *T. serialis*, *T. brauni*, and *T. glomerata* (11). Human coenurosis usually involves the CNS, muscles, or subcutaneous tissues (12).

Usually, cerebral coenurosis is caused by the larval stage of *T. multiceps multiceps*. Although coenurosis has been frequently recorded in sheep and goats in Iran (13-16), human cases have not been reported so far.

Unlike the unilocular echinococcal cyst of the brain, no brood capsules are found in *Coenurus* in the brain (11). The symptoms of cerebral coenurosis resemble those of CCE and neurocysticercosis; hence, the disease goes undiagnosed (11, 17). The diagnostic methods are crucial in differentiating coenurosis from CCE, and this entity should be included in the differential diagnosis, especially in countries where these diseases are prevalent and endemic. Although coenuri are easily distinguished morphologically from cysticerci and echinococcal cysts based on the numbers and characteristics of their protoscoleces, larval hook morphology has been considered a valid criterion for identification (18, 19). When protoscoleces are absent or not identifiable, *Coenurus* could be distinguished from echinococcal cysts by the lack of the characteristic acellular, laminated membrane. However, racemose (acephalic) Cysticercus often cannot be differentiated from *Coenurus* because the thin cyst membranes of the two cysts are similar (20). Apart from imaging methods, there is no clinical way of differentiating cerebral coenurosis from cysticercosis or echinococcosis. Moreover, in brain infections, more than 50% of coenuri are without protoscoleces, and histopathological sections are not helping to differentiate these cysts from cerebral echinococcal cysts (21). Therefore, morphology alone is not adequate for the differentiation of *Coenurus* and echinococcal cysts (22, 23), and in such cases, a molecular study on clinical samples of unfertile cerebral cysts is necessary.

Regarding the high prevalence of *T. multiceps*

in carnivores in Iran (24), a possibility for human infection with *Coenurus* is predicted. Due to the transmission of both cystic echinococcosis and coenurosis in Iran, and the fact that over 50% of coenuri in humans are sterile (acephalocysts or without protoscoleces), there is a need to characterize the causative agents of brain cysts caused by the parasites to determine the transmission patterns for the planning of prevention and control programs in human and animal communities. The present study aimed to differentiate CCE from coenurosis using morphometric analysis of larval rostellar hooks and the PCR-sequencing methods.

Material and Methods

Sample collection

Four humans cerebral echinococcal cysts kept in 70% ethanol were collected from Ahvaz University of Medical Sciences, Iran (7). Three cerebral coenuri were collected from sheep, one from Tehran University of Medical Sciences, one from Kerman University of Medical Sciences, and one from Shiraz University, Iran. In addition, a non-cerebral *Coenurus* isolated from sheep muscles in Shiraz, Fars Province, Iran, collected in 2011, kept in 70% ethanol was used for comparison. Two cerebral coenuri which were collected from Tehran and Shiraz preserved in formaldehyde used for morphological criteria and we did not achieve to extract their DNA. However, DNA was extracted from cerebral coenuri which was collected from Kerman.

Morphometric analysis

Three protoscoleces were separated from each isolate, stained, cleared, and mounted using Formaldehyde Alcohol Azocarmine Lactophenol (FAAL) on a glass slide with sufficient cover-slip pressure to cause the hooks to spread out without damaging them (25). Using micrometry, three large and three small hooks from each protoscolex were measured for several morphometric characters, including the total number of rostellar hooks per protoscolex (NH), total length of large hooks (LTL), total length of small hooks (STL), large hook blade length (LBL), small hook blade length (SBL), large hook handle length (LHL), small hook handle length (SHL), the ratio of blade length to total length of large hook (LBL/LTL), and the ratio of blade length to total length of small hook (SBL/STL) (Fig. 1) (18).

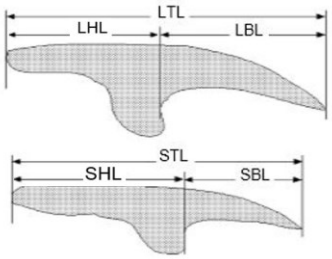

Fig. 1: Diagrammatic representation of hook dimensions as used in this study. Total length of large hooks (LTL), total length of small hooks (STL), large hook blade length (LBL), small hook blade length (SBL), large hook handle length (LHL), small hook handle length (SHL)

PCR-sequencing method

The human cerebral echinococcal cysts were investigated using the PCR-sequencing method (7). The genomic DNA from four isolates of coenuri (three cerebral coenuri and a non-cerebral *Coenurus*) was extracted using Qiagen, QIAamp ® DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instruc-

tions. The forward primer (MS1: 5´-CGTAGGTATGTTGGTTTGTTTGGT-3´) and the reverse primer (MS2: 5´-CCATAATCAAATGGCGTACGAT-3´) (26).

The PCR products were sequenced in two directions using the same forward and reverse primers used in the PCR by the Sanger sequencing method. The nad1 gene sequence results were edited by the Geneious software (www.geneious.com), and the consensus sequences were compared with the reference sequences available in GenBank using BLAST (http://www.ncbi.nlm.nih.gov/) for identification. Nucleotides of 334 bp from cerebral echinococcal cysts and 332 bp from *T. multiceps* and *T. multiceps gaigeri* were used in the phylogenetic tree. A phylogenetic tree was constructed with sequences obtained in the present study along with the reference sequences deposited in GenBank using the maximum likelihood method and Tamura-Nei model in the Molecular Evolutionary Genetic Analysis software (MEGA 5.0) with 1000 bootstrap replicates to determine the robustness of the finding.

Statistical analysis

Morphometric data were analysed using SPSS software ver. 23 (IBM Corp., Armonk, NY, USA). Scatter chart was drawn based on large hook blade length versus large hook total length and small hook blade length versus small hook total length for *C. cerebralis* and CCE samples using Excel 2013 software.

Results

Morphometric results

One isolate out of four human cerebral echinococcal cysts and the four isolates of coenuri were investigated morphologically. Three isolates of cerebral echinococcal cysts were sterile, and did not have any protoscolex. Three protoscoleces containing one row of large hooks and one row of small hooks were randomly selected from each fertile isolate. Three large hooks and three small hooks from each protoscolex isolated from cerebral echinococcal cysts, *C. cerebralis* and non-cerebral *Coenurus*, were measured (Fig. 2).

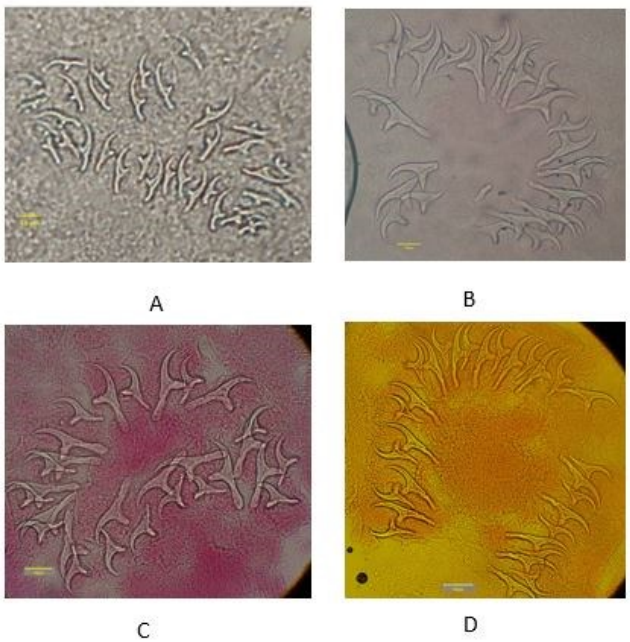

Fig. 2: Rostellar hooks of protoscolex obtained from cerebral cystic echinococcosis (40× Scale bar 10 µm (A), *Coenurus cerebralis* (20× Scale bar 50 µm) (B, C) and non-cerebral coenurosis (20×) (D Scale bar 50 µm)

Different parameters of larval hooks measurements of protoscoleces isolated from *C. cerebralis* and non-cerebral coenuri are presented in Table 1.

Table 2 shows the comparison of *C. cerebralis* and cerebral echinococcal cysts based on the number and size of rostellar hooks. The total size of the large hooks (Fig. 3) and small hooks (Fig. 4) of *C. cerebralis* was larger than that of the cerebral echinococcal cyst. These parameters demonstrated the morphologically significant differences between the *C. cerebralis* and cerebral echinococcal cyst (*P*-value<0.05).

Table 1: Different parameters of larval hooks measurements of protoscoleces isolated from *Coenurus cerebralis* and non-cerebral coenuri

Parameters	*Coenurus cerebralis* Mean ± S.D.	Non-cerebral coenuri Mean ± S.D.	P-value
Total number of hooks (NH)	27.3 ± 1.4	30.0 ± 0.0	0.009*
Large hook total length (LTL) (µm)	163.9 ± 11.3	155.6 ± 3.9	0.05*
Large hook blade length (LBL) (µm)	69.4 ± 4.7	68.9 ± 2.2	0.7*
Large hook handle length (LHL) (µm)	94.4 ± 8.5	86.7 ± 3.5	0.01*
LBL/LTL	0.42 ± 0.02	0.44 ± 0.05	0.8*
Small hook total length (STL) (µm)	112.0 ± 11.3	115.6 ± 5.3	0.7
Small hook blade length (SBL) (µm)	51.5 ± 5.5	52.2 ± 3.6	0.7
Small hook handle length (SHL) (µm)	60.6 ± 8.5	63.3 ± 3.5	0.5*
SBL/STL	0.46 ± 0.04	0.47 ± 0.05	0.7

* *P*-value was calculated by Mann-Whitney Test

Table 2: Comparison of cerebral cystic echinococcosis and *Coenurus cerebralis* based on the number and size of rostellar hooks

Parameters	Cerebral cystic echinococcosis human origin Mean ± S.D.	*Coenurus cerebralis* sheep origin Mean ± S.D.	*P*-value
Total number of hooks (NH)	34.7 ± 3.1	27.3± 1.4	0.000*
Large hook total length (LTL) (µm)	24.4 ± 1.1	163.9 ± 11.3	0.000*
Large hook blade length (LBL) (µm)	15.0 ± 1.7	69.4 ± 4.7	0.000*
Large hook handle length (LHL) (µm)	9.4 ± 1.1	94.4 ± 8.5	0.000*
LBL/LTL	0.60 ± 0.05	0.42 ± 0.02	0.000*
Small hook total length (STL) (µm)	17.5 ± 0.0	112.0 ± 11.3	0.000*
Small hook blade length (SBL) (µm)	10.0 ± 0.0	51.5 ± 5.5	0.000*
Small hook handle length (SHL) (µm)	7.5 ± 0.0	60.6 ± 8.5	0.000*
SBL/STL	0.57± 0.00	0.46 ± 0.04	0.000*

* *P*-value was calculated by Mann-Whitney Test

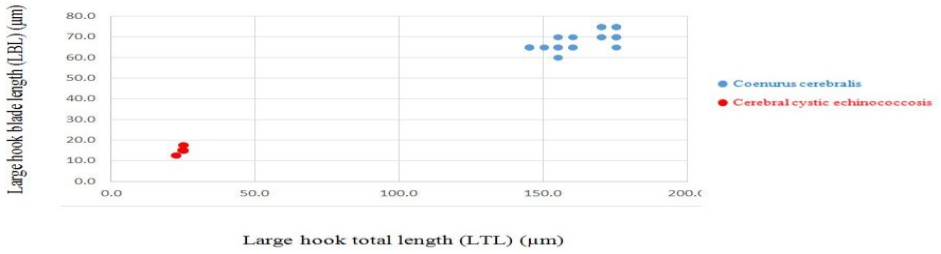

Fig. 3: Scatter chart of large hook blade length versus large hook total length for *C. cerebralis* and cerebral cystic echinococcosis. Each point represents the mean values of these two variables measured on three large hooks from each of three protoscoleces from every isolate

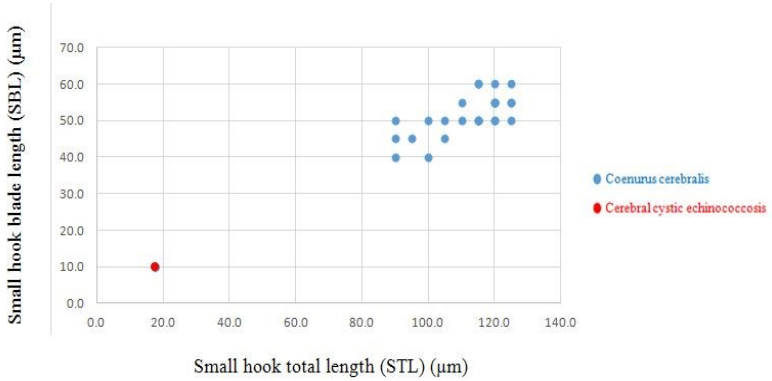

Fig. 4: Scatter chart of small hook blade length versus small hook total length for *C. cerebralis* and cerebral cystic echinococcosis. Each point represents the mean values of these two variables measured on three large hooks from each of three protoscoleces from every isolate

Molecular results

Three isolates out of four humans cerebral echinococcal cysts were identified as *E. canadensis,* G6 genotype (Accession no. JN621320-JN621322), and one CEC isolate had unsuccessful PCR (7). The nad1 sequences of the cerebral echinococcal cysts were quite similar and had 100% homology with the *E. canadensis* G6 genotype isolated from camels in Kenya (KX010873) (Fig. 5).

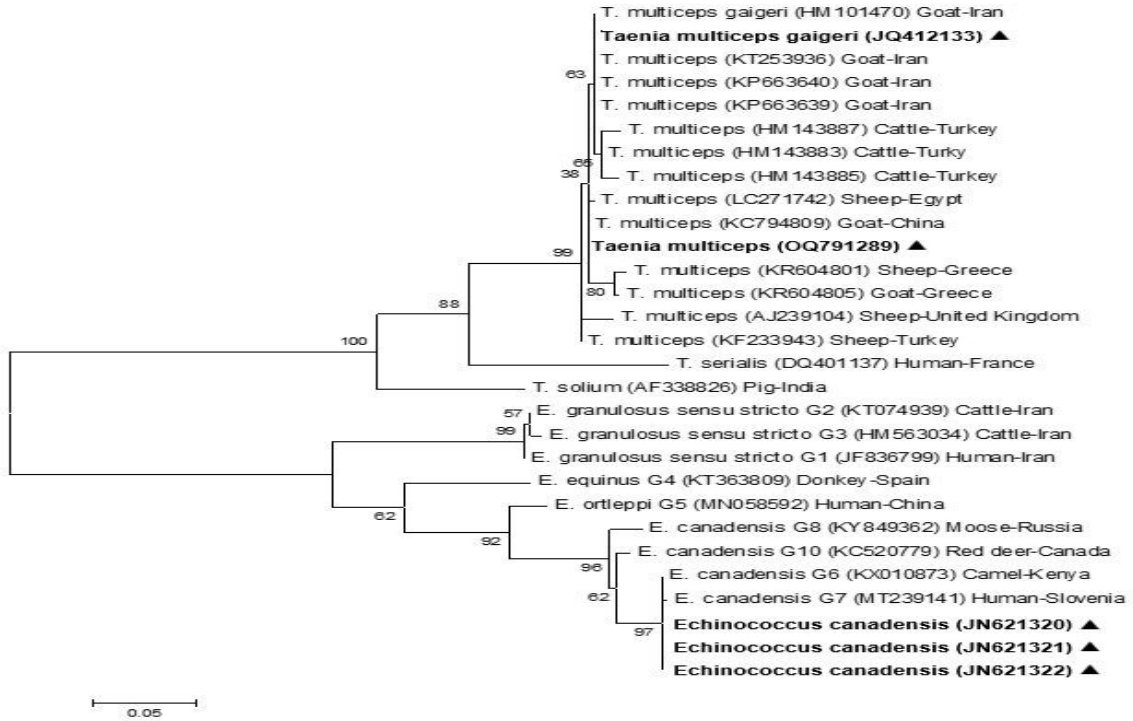

Fig. 5: Phylogenetic analysis of the nad1 sequences of *Taenia multiceps* and *T. multiceps gaigeri* isolates obtained in this study and *Echinococcus canadensis* isolates obtained in other study (7) (black upward-pointing triangle) and reference sequences retrieved from GenBank.
The tree was constructed using the Maximum Likelihood method. *Taenia solium* (AN: EF076753) was used as the out-group

An approximately 400 bp band was amplified from the isolates of *C. cerebralis* and non-cerebral *Coenurus* after the PCR for the mitochondrial nad1 gene. One isolate of *C. cerebralis* and one isolate of non-cerebral *Coenurus* were also sequenced. Due to preservation in formaldehyde, the PCR for two more other cerebral *C. cerebralis* isolates, one from Tehran and one from Shiraz was unsuccessful. Sequence analysis was performed, and the edited sequences were compared with other available sequences in GenBank using BLAST. The cerebral *Coenurus* and the non-cerebral *Coenurus* were identified as *Taenia multiceps* and *T. multiceps gaigeri*, respectively. The consensus sequences of cerebral *Coenurus* and non-cerebral *Coenurus* obtained in this study were deposited in the GenBank database with the accession numbers OQ791289 and JQ412133, respectively.

According to phylogenetic analysis based on the nad1 gene, the sequence of the isolate of *T. multiceps* obtained in the present study (OQ791289) showed 100% homology with the reference sequence of *T. multiceps* from China (KC794809). The sequence of *T. multiceps gaigeri* (JQ412133) isolated in this study was similar to that of *T. multiceps gaigeri* (HM101470) and *T. multiceps* isolates (KT253936, KP663639, and KP663640) isolated from goats in Iran (Fig. 5).

Intra-species variation within the nad1 sequences of the *T. multiceps* obtained in this study and other isolates of *T. multiceps* existing in GenBank amounted to 0%-3.8%.

Discussion

CE occurs commonly in the liver or lungs, and cerebral cystic echinococcosis is rare (4). Coenurosis is a rare zoonotic disease that requires a differential diagnosis from CCE (11). Usually, the diagnosis and management of human cestode infection is based on a combination of clinical symptoms, radiological, morphological, serological, molecular and proteomics methods (25-29). The symptoms of cerebral coenurosis are similar to those of CCE, and serological methods are not effective in the diagnosis of the disease because of cross-reaction with other taeniid cestodes. The diagnosis of human coenurosis is based on various radiological methods, such as CT scan, but it may be misdiagnosed from CCE. The differentiation between these two diseases is very important, and the surgical removal of the cyst and morphological examination are the definitive methods for the differential diagnosis of these cystic lesions (17). Usually, cerebral echinococcal cysts are differentiated from *Coenurus* cysts based on their rostellar hook number, size, and shape. In many human cases (over 50%) *Coenurus* cysts are sterile, without the presence of protoscolices. There is no clinical way of differentiating infection with this parasite from cases of cysticercosis or echinococcosis (21). Hence, molecular methods can be used to identify the brain cysts, especially in endemic areas for two diseases. Iran is known as one of the endemic countries for both *E. granulosus* and *T. multiceps* (16, 27, 30). In the current study, both morphometric and molecular methods were used to differentiate cerebral coenurosis from CCE.

Nine morphometric indices of the protoscoleces isolated from cerebral echinococcal cyst were measured. The total number of rostellar hooks in cerebral echinococcal cysts was 32-38, which was similar to the other studies, but the total length of large and small hooks in cerebral echinococcal cysts was shorter than that of hooks originated from camels in other studies (31, 32). Larval hook morphology could be used for differentiating G1-G3 and G6 genotypes of *E. granulosus* (18), while some reports showed that morphometric analysis alone is not enough for the determination of *E. granulosus* strains and a molecular method should be used (32).

The PCR-sequencing method has been used over recent years for species identification, classification, and phylogenetic analysis of

family Taeniidae (31). Since the morphological identification of some species is difficult, some molecular methods, such as PCR-sequencing using nuclear and mitochondrial markers, have been used for accurate identification and differentiation of taeniid cestodes (7, 33-35). For the identification of *E. granulosus* genotypes, different methods have been used, and molecular methods have divided *E. granulosus sensu lato* into 10 distinct genotypes (G1-G10) in different regions of the world (6). The G1-G3 (*E. granulosus sensu stricto*) and G6 genotypes (*E. canadensis*) were reported as the most common species of human CE in different geographic areas of Iran (7, 18, 26, 27). The cerebral echinococcal cysts investigated in this study has been identified as the G6 genotype (*E. canadensis*) reported that the genotype G6 has a special tendency toward the brain in humans (7). Phylogenetic analysis of the nad1 sequence of *E. canadensis* showed that there was 100% similarity between *E. canadensis* obtained in this study and the isolate collected from camels in Kenya.

PCR-sequencing using the mitochondrial nad1 gene has been used for the characterization of *Coenurus* in sheep from different geographic areas of Italy (36). The mitochondrial nad1 gene by PCR-sequencing has also been used for molecular identification of *Coenurus* (34). In the current study, genomic DNA was extracted from the isolates of *Coenurus*, and cerebral *Coenurus* was identified as *T. multiceps*. The non-cerebral *Coenurus* was investigated molecularly and identified as *T. multiceps gaigeri* by amplification of the nad1 gene, which was similar to another study (37). The larval stages of *T. multiceps*, are considered the causative agent for both cerebral and non-cerebral coenurosis, while the larval stages of *T. multiceps gaigeri* are regarded as the causative agent of non-cerebral coenurosis (20).

The non-cerebral *Coenurus* identified as *T. multiceps gaigeri* had two rows of rostellar hooks with 30 small and large hooks, and the large and small hook total lengths were 150-160 µm and 115-125 µm, respectively. Our results are in agreement with the other published morphometric study (37). Although non-cerebral *Coenurus* is morphologically similar to *C. cerebralis*, the mean total number of protoscolex hooks of *C. cerebralis* and non-cerebral *Coenurus* was 27.3 and 30, respectively. The average total length of the large hooks of *C. cerebralis* and non-cerebral *Coenurus* was 163.9 µm and 155.6 µm, respectively, and the difference was statistically significant. The morphometric results of our study regarding *C. cerebralis* were consistent with another results (38). In contrast to our study, Akbari et al. reported no significant difference between cerebral and non-cerebral *Coenurus* according to the number of large and small hooks (19).

According to the data, all nine morphometric indices of large and small hooks from *C. cerebralis* had significant differences with those of cerebral echinococcal cysts. However, the problem is the differential diagnosis of *Coenurus* and cerebral echinococcal cysts, especially in the sterile situation of coenorosis infection, whose frequency reaches as high as 50% of the brain cysts (21). Therefore, the application of molecular methods rather than histopathological exams is necessary and should be used to identify different cerebral sterile, especially in endemic regions of *E. granulosus s.l.* and *T. multiceps*.

Conclusion

Cerebral coenurosis could be differentiated from CCE based on morphometric and molecular analysis of brain cysts. Despite the sample size limitation, in this study, all nine morphometric indices significantly different between *C. cerebralis* and cerebral echinococcal cyst, therefore, morphometric analysis is a useful tool for their differential diagnosis. However, given the similarities between sterile cases of cerebral echinococcal cysts and cerebral coenuri, and the high frequency of sterile *Coenurus* cysts, molecular methods are recommended to accurately distinguish cerebral echinococcal cyst from *C. cerebralis*, especially

in regions where both types of cysts are common.

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Conflict of interest

The authors declare that there is no conflict of interests.

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