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Degree of calcification and cyst activity in hepatic cystic echinococcosis in humans

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ABSTRACT

To evaluate the relationship between cyst activity and calcification degree in cystic echinococcosis (CE) in humans, 99 hepatic cysts at successive stages of involution, surgically excised from 72 Sardinian patients, have been analyzed. Cysts were classified into 4 groups according to calcification extent: CALC 0 (no calcification); CALC 1 (scattered punctate calcifications); CALC 2 (large coarse segmental/partial calcifications); CALC 3 (complete or nearly complete circumferential ring of calcification up to thick wall of osseous consistency/calcified content of cyst). In addition the possible correlation with antibody response has been explored analyzing IgG1, IgG4 and IgE produced against somatic PSCAg. Results showed that calcification is not restricted to the inactive WHO cyst types CE4 and CE5, but occurs to a varying extent in all morphotypes of metacestode, from active *classic unilocular* or *multivesicular* cysts to the more complicated and highly degenerate stages, where cyst wall appears massively calcified. Prevalence of calcification increases with progression of cyst degenerative process, but is not synonymous with parasite inactivity and can be misleading as signs of calcification may coexist with still metabolically active cysts. On the contrary, detection of entirely firmly solidified content seems a reliable indication of cyst inactivity. IgG4 is the dominant isotype associated particularly with the evolutive phase. Positive rates and OD levels, higher in active vs inactive stages, are stable or increase slightly in weakly and moderately calcified cysts (CALC 1/CALC 2), compared to non-calcified ones (CALC 0), strongly decreasing in highly calcified forms (CALC 3). In conclusion, evaluation of calcification extent may be pertinent for staging CE, and immunological tests, particularly for IgG4, and IgE may help to better define cyst activity.

1. Introduction

Cystic echinococcosis (CE) is a widespread chronic helminthic disease caused by infection with the larval form of the cestode *Echinococcus granulosus*.

As is well known, the CE life cycle is based on a predator-prey relationship between the definitive host that harbors the adult worm, and the intermediate hosts that harbor the larval stage of the parasite. After the first *in vitro* culture studies (Smyth and Davies, 1974) showing the differential growth requirements between parasite protoscoleces of sheep vs. horse origin, different biological variants of *E. granulosus*, which utilize particular ungulates as intermediate hosts (water buffalo, Tasmanian sheep, cattle, camels, pigs, cervids), have been described, according to host specificity. Molecular studies shed light on this field, supporting the strain definition and allowing to identify over time a number of genotypes (G1–10), until the study by Nakao et al. (2007) based on mitochondrial genome sequences reconstructed the phylogenetic relationships indicating that *E. granulosus* sensu lato (s.l.) is a cryptic species complex. According to current taxonomic revision *E. granulosus* s.l. encompasses five species: *E. granulosus* sensu stricto (s.s.) (G1-G3), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5), *Echinococcus canadensis* (G6-G10) and *Echinococcus felidis* (Alvarez Rojas et al., 2014; Ito et al., 2017). *E. granulosus* s.s. (G1) is the major causative organism of human CE (more than 88% of cases according to Alvarez Rojas et al., 2014) where sheep are grazed with dogs, but in some regions where camels and other livestock including cattle, pigs, and goats are bred, G6/7 genotypes may also be responsible for human CE (about 11%: Alvarez Rojas et al., 2014).

Due to its life cycle the zoonosis is endemic in many regions, as in the Mediterranean Basin. In Sardinia, an Italian island in the center of the Mediterranean where sheep raising has been common practice since ancient times and a great number of dogs (“sheep-”, “community-” and

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Table 1
Number of CE Sardinian patients grouped according to age and gender.

	Age groups (years)								
	0 – 10	11–20	21–30	31–40	41–50	51–60	61–70	> 70	**TOTAL**
M	1	2	4	7	10	10	5	4	**43**
F	0	1	1	7	4	6	10	0	**29**
TOTAL	**1**	**3**	**5**	**14**	**14**	**16**	**15**	**4**	**72**

"stray-" dogs) are present, CE is historically highly widespread both in animals and in humans (Ferretti et al., 1977; Conchedda et al., 1985, 1997, 2002; Gabriele et al., 2004a, 2004b). Despite improvements registered in the last decades, the most recent epidemiologic data show a CE prevalence of over 65–75% in sheep (Conchedda et al., 2012), and a mean annual incidence rate of human CE cases of 6.6 per 100,000 inhabitants (Conchedda et al., 2010).

As is well known, CE in humans is typically a chronic disease characterized by the gradual and slow development and size increase of the cyst, in relation to compactness of the parenchyma of the organ involved. In particular in the liver, the most affected organ, CE metacestode can remain asymptomatic for decades, until the cyst grows to an extent that triggers clinical (even non-specific) signs. The natural history of CE in humans has demonstrated that, because of the long latency, the metacestode goes through a series of complex transformation processes that cause degeneration and may ultimately result in the death of the parasite (Bortoletti et al., 2002, 2004, 2013).

Following the introduction of ultrasound (US) as a routine imaging diagnostic tool, various attempts have been made to characterize human cysts with respect to this process of transformation. Gharbi's classification, the first and most enduring attempt with five classes of cyst types (Gharbi et al., 1981), represents the basis for all subsequent ultrasound categorization (Lewall and McCorkell, 1985; Caremani et al., 1997). Finally in 2001, in the attempt to unify and simplify previous categorizations, a standardized CE ultrasound classification was proposed by the WHO Informal Working Group on Echinococcosis comprising six types (CL, CE1, CE2, CE3, CE4, and CE5), from a very early stage of development to involute, necrotic inactive parasites (Pawlowski et al., 2001; WHO-IWGE, 2003). This may provide a useful basis for the clinical management of CE, helping to select the appropriate treatment from among surgery, percutaneous treatment (PAIR), chemotherapy with benzimidazoles or the most recent "wait and see" approach.

In this respect, the in-depth morphostructural study of excised CE cysts, with analysis of the transformations that can take place over time in larval forms in humans (Bortoletti et al., 2002, 2004, 2013) has made it possible to characterize more precisely the various morphofunctional types of CE cysts, from young and viable to inactive and dead cysts. In the meantime, a study on the immunoproteome profile during CE progression has recently shown that specific immunodominant epitopes change as the disease progresses (Ahn et al., 2015).

Calcification, generally observed by radiography in 20%-30% of CE cysts (Pedrosa et al., 2000) is usually regarded as the terminal stage of parasite degeneration during its natural evolution in humans, and is believed to be an index of cyst inactivity. The potential importance of calcification in relation to cyst activity has recently prompted research in China, where CE is widespread, to explore the gene (biomarkers) correlated to cyst calcification by analyzing relative expressions of galecitin-4 (LGALS4) and acid ceramidase (ASAH1) in patients with calcified and non-calcified cysts, with a view to developing reliable tools for improving viability assessment (Yin et al., 2016).

In the present research, the availability of surgically removed intact hepatic CE cysts of varying morphotypes, together with data from ultrasonography (US) and computed tomography (CT) reports, made it possible to investigate the progress of calcification during the process of transformation and degenerative evolution of the parasite.

The aim of this study was precisely to compare calcification with cyst viability by direct insight into the morphofunctional status of the cysts, also exploring the possible correlation with antibody response. The research, entailing *de visu* analysis of the whole cysts in parasitological terms, provides an accurate picture of the parasite and enables to exactly verify, by direct observation, whether cysts of the studied sample were actually alive and viable. The biological analyses that focused on investigating the relationship between degree of calcification and metacestode activity in cysts at varying stages of involution, may contribute to providing further insight into the natural history of CE that could help clinicians in the choice of appropriate treatment.

2. Materials and methods

2.1. Patients and cyst classification

The study concerned a total of 99 hepatic cysts, classified according to US WHO groups (CE1-CE5) (Pawlowski et al., 2001; WHO-IWGE, 2003) surgically excised at the Clinica Chirurgica, Cagliari University, from 72 Sardinian patients, 43 males and 29 females (male-to-female ratio = 1.48), with mean age of 49 years (S.D. 14.5; range 15–78 years) (Table 1).

The cysts were surgically removed by physicians on assessment of patients' clinical conditions and sent to our Parasitology Laboratory for routine morphostructural and biological examination (Nardello et al., 2006). In no way were the cysts excised *in toto* for the purposes of the present study. All data were analyzed anonymously.

On the basis of the study on the natural history of CE infection in humans (Bortoletti et al., 2002, 2004, 2013) hepatic cysts, the majority excised *in toto* by total pericistectomy, were grouped into different morphological types: *unilocular, multivesicular, transitional, hyperlaminated, hyperlaminated-caseous, hyperlaminated-granular, hyperlaminated-gelatinous, caseous* and *acephalous* cysts. In detail, shape, size, cyst wall status and cavity content of excised specimens were examined. As already described (Bortoletti et al., 2002; Conchedda et al., 2016) cyst fertility was carefully evaluated by analyzing the sediment within the cyst content for the presence of hydatid sand, and by gently scraping in saline the walls of daughter cysts and any sheets of laminar tissue present in the cyst cavity, to harvest adhering brood capsules, protoscoleces (PSC) or residual PSC. Viability was assessed by 0.1% methylene blue exclusion test and by checking motility of flame cells (Casado et al., 1986).

2.1.1. Calcification

The extent of calcification of the cyst wall and content was evaluated by direct morphostructural examination of collected specimens, also comparing data with ultrasonography (US) and computed tomography (CT) reports. The cysts were classed into 4 groups according to the degree of calcification: CALC 0 (no calcification observable); CALC 1 (scattered punctate calcifications); CALC 2 (large coarse segmental/partial calcifications); CALC 3 (complete or nearly complete circumferential ring of calcification up to thick wall of osseous consistency/calcified content of cyst).