ECHINOCOCCUS GRANULOSUS: FIRST REPORT OF MICROCYSTS FORMATION FROM PROTOSCOLECES OF CATTLE ORIGIN USING THE *IN VITRO* VESICULAR CULTURE TECHNIQUE

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Summary:

The aim of this work was the achievement of microcysts formation from protoscoleces of *E. granulosus* of cattle origin using the *in vitro* vesicular culture technique. Vesiculated protoscoleces and protoscoleces with posterior bladders appeared during the first week of incubation. After 14 days of culture, a laminated layer appeared like a fine membrane in one of the extremes of the protoscoleces. On day 20, some microcysts with a complete laminated layer were observed. By day 48, microcysts completely developed could be observed. This is the first study where microcysts formation was obtained using protoscoleces of *E. granulosus* of cattle origin.

KEY WORDS: *Echinococcus granulosus*, cystic echinococcosis, protoscoleces, *in vitro* culture.

Résumé : *ECHINOCOCCUS GRANULOSUS* : PREMIER RAPPORT D'OBTENTION DE MICROKYSTES DE PROTOSCOLEX D'ORIGINE BOVINE PAR LA TECHNIQUE DE CULTURE *IN VITRO*

L'objectif de notre étude était l'obtention de microkystes de protoscolex d'*E. granulosus* d'origine bovine en utilisant la technique de culture *in vitro*. Des protoscolex vésiculeux et des protoscolex avec une vésicule postérieure sont apparus durant la première semaine d'incubation. Après 14 jours de culture, la membrane laminaire apparaît comme une fine membrane à l'une des extrémités des protoscolex. Autour du jour 20, quelques microkystes avec une membrane laminaire complète sont observés. Au jour 48, des microkystes complètement développés sont obtenus. C'est la première fois que l'on rapporte l'obtention de microkystes de protoscolex d'*E. granulosus* d'origine bovine.

MOTS CLÉS : *Echinococcus granulosus*, échinococcose cystique, protoscolex, culture *in vitro*.

Hydatid disease is caused by the larval stage of *Echinococcus granulosus*, which can establish itself in a wide range of intermediate hosts including cattle, sheep, pigs, horses and humans. These hosts become infected by ingestion of eggs in contaminated food or water (Breijo *et al.*, 1998).

Protoscoleces have a dual potential for development; on one hand, they are the infective stage of the adult parasite in the definitive host, on the other hand, they are capable of reversibly developing into cysts in the intermediate host when released by accidental rupture of the mother cyst (secondary hydatidosis) (Lightowers, 1990).

The first attempts of *in vitro* cultivation of *E. granulosus* protoscoleces were made by Dévé (1901, 1902). However these assays were unsuccessful because protoscoleces died after 28 days in *in vitro* conditions. The first successful *in vitro* cultivation in a cystic direc-

tion was reported by Smyth (1962). Since then, a significant progress was made using a variety of media and conditions (Benex, 1968; Brudnjak *et al.*, 1970; Heath & Osborn, 1976; Rogan & Richards, 1986; Casado *et al.*, 1986; Rodriguez-Caabeiro & Casado, 1988; Casado & Rodriguez-Caabeiro, 1988a & b).

Rodriguez-Caabeiro & Casado (1988) worked with hydatid cysts of sheep origin. They observed microcysts formation after 15 days. Ponce Gordo & Cuesta Bandera (1997) worked with cysts of sheep, cattle, horse, pig and human origin. Working with this material, they found differences not only in the time of microcysts formation, but also in the development process. They obtained microcysts from protoscoleces of sheep, pig, horse and human origin. The development to microcysts could not be achieved with material of cattle origin. Their initial development was normal but in no case microcysts finally formed.

The *in vitro* cultivation of *E. granulosus* protoscoleces allows to obtain information applicable to the hydatid disease control programmes and in biochemical, immunological and treatment studies (Casado *et al.*, 1986).

The aim of this work was the achievement of microcysts formation from protoscoleces of *E. granulosus* of cattle origin using the *in vitro* vesicular culture technique.

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Fig. 1. – Protoscoleces obtained of lung bovine cysts. The typical shape of protoscoleces is observed, hooks (H) and calcareous corpuscles (CC). Dead stained protoscolec (white arrow). Methylene blue exclusion test 1:10,000.

Fig. 2. – Evaginated protoscolec.

Fig. 3. – Vesiculated protoscolec. An evaginated protoscolec showing a posterior bladder is observed (arrow).

Fig. 4. – Vesiculating protoscolec (three days of culture). Note the increment in the size.

Fig. 5. – Microcyst showing laminated layer (20 days of culture) (arrow).

Fig. 6. – Microcyst completely developed (48 days of culture).

MATERIALS AND METHODS

Hydatid cysts from naturally infected bovine lungs and livers were obtained from two abattoirs located in the southeast of Buenos Aires province, Argentina. Protoscoleces were removed from cysts by aseptic techniques and washed several times with phosphate-buffered saline (PBS) pH 7.2. Viability was assessed by the methylene blue exclusion test (Casado *et al.*, 1986) or by muscular movements, morphological perfectness of the whole body, and motility of flame cells. Groups of 1,500 viable and free protoscoleces were transferred to Leighton tubes, one to five replicates per sample, depending on quantity of available protoscoleces. The culture medium was 199 (Gibco) containing 100 IU penicillin, 100 µg/ml streptomycin, 4 mg/ml glucose and 20 % (v/v) foetal calf serum. Cultures were maintained at 37°C and the medium was changed every three-four days. A total of 10 samples (seven from lung and three from liver) was cultured. Development was followed microscopically every day. Development time until cysts was determined in days. The data recorded for each sample correspond to the moment when a stage was reached by the most advanced individuals of the culture.

RESULTS

At the beginning of the culture protoscoleces were invaginated (Fig. 1). After 48-72 hours of incubation some evaginated protoscoleces could be observed (Fig. 2). The amount of them is variable in the different cultures, between 5 and 20 %, but it never reached 100 %.

Vesiculated protoscoleces and protoscoleces with posterior bladders (Figs 3 & 4) appeared during the first week of incubation. Moreover, a considerable increment in the size of them could be observed.

After 14 days of culture, a laminated layer appeared like a fine membrane in one of the extremes of the protoscoleces. Seventy percent of all samples reached the microcyst formation (57.1 % of lung and 100 % of liver samples). On day 20, some microcysts with a complete laminated layer were observed (Fig. 5). Some samples showed microcyst formation between 24-38 days. By day 48, microcysts completely developed could be observed (Fig. 6). In all cultures, the total number of microcyst formed was approximately 1.6 % of the number of initial cultured protoscoleces. Some cultures could be maintained until day 95.

DISCUSSION

This is the first study where microcysts formation was obtained using protoscoleces of *E. granulosus* of cattle origin.

As Smyth described (1962), we found two patterns of cystic development *in vitro*. In the first, some protoscoleces became swollen or vesicular within a few days of culture and grew into thin walled cysts. In the non-vesicular pattern of development, the protoscoleces each develop a posterior bladder which increased substantially in size; eventually a spherical cyst formed and this became enveloped by a laminated layer. Such cysts were morphologically indistinguishable from cysts that developed from vesicular protoscoleces. This also coincides with the works done by Casado & Rodríguez-Caabeiro (1988a) and Ponce Gordo & Cuesta Bandera (1997).

In this study, the time for microcyst formation in days was determined between 20 and 38. Working with sheep samples, Ponce Gordo & Cuesta Bandera (1997) observed microcyst formation between 19 and 37 days and Casado & Rodríguez-Caabeiro (1988a & b) at day 15. By the other hand, the results of this work with cattle samples are clearly different from those indicated by Ponce Gordo & Cuesta Bandera (1997). A possible explanation could be the use of a different culture medium. Another reason that could explain the difference is the possibility of a different genotype. The material used for the *in vitro* culture was not yet characterized by molecular techniques to determined the involved strains, but only the G1 strain was identified in cattle isolates of Buenos Aires Province (Rosenzvit *et al.*, 1999, 2002; Kamenetzky *et al.*, 2002).

The obtention of microcyst of *E. granulosus* should allow us to test *in vitro* different new anthelmintic drugs for the human treatment of hydatid disease.

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