# Short term culture of protoscoleces to obtain excretory-secretory proteins of *Echinococcus granulosus*.

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Abstract: A maintenance medium for protoscoleces of *Echinococcus granulosus* to obtain excretory-secretory antigens for further immunochemical characterization and evaluation of their potential immunodiagnostic use is described. This medium consists of PBS buffer with 10% dextrose and antibiotics. Cultures of protoscoleces collected from sheep liver and lung hydatid cysts were carried out. Protoscoleces were washed with and without the addition of pepsin to the washing solution. Viability curves in the various assays performed revealed that the survival of the protoscoleces obtained from lung cysts was longer than that for liver cysts. Pepsin-treated protoscoleces were less viable than those washed through simple serial PBS buffer sedimentation. Protein concentration curves in the cultures failed to show marked differences between protoscoleces from liver and lung hydatid cysts. However, treatment with pepsin was shown to induce a lower protein synthesis as compared to the simple washing with PBS buffer. From the analysis of the data obtained, it is concluded that the first 50 hours of cultivation is the optimal period to obtain excretory-secretory proteins from the protoscoleces of *E. granulosus*.

Keywords: *Echinococcus granulosus*, echinococcosis, hydatid disease, excretory-secretory antigens, parasite cultures.

Resumen: En este trabajo se describe un medio de mantenimiento de protoescólex de *Echinococcus granulosus* para la obtención de antígenos de excreción-secreción con fines inmunodiagnósticos. Este medio está constituido por tampón PBS con glucosa al 10% y antibióticos. Se llevaron a cabo experiencias con protoescólex de procedencia ovina hepática y pulmonar, y se compararon dos métodos de lavado para los mismos: la decantación en tampón PBS y el tratamiento con pepsina. Las curvas de supervivencia de los diferentes ensayos realizados mostraron una mayor longevidad de los protoescólex de origen pulmonar con respecto a los de procedencia hepática, mientras que los protoescólex tratados con pepsina poseían una menor viabilidad que los lavados mediante decantaciones sucesivas con tampón PBS. Las curvas de concentración de proteínas de los diferentes cultivos revelaron que no existían diferencias notables entre los protoescólex hepáticos y pulmonares. Sin embargo se comprobó que el tratamiento con pepsina inducía una menor síntesis proteica en relación con el lavado con tampón PBS. Del análisis de estos datos se determinó que el periodo óptimo para la obtención de antígenos de excreción-secreción de protoescólex de *E. granulosus* era el correspondiente a las primeras 50 horas de mantenimiento de los protoescólex.

Palabras clave: *Echinococcus granulosus*, equinococosis, antígenos excreción-secreción, cultivos de parásitos.

## 1. Introduction

The diagnosis of human hydatid disease is mainly based on the detection of antibodies by using serological methods of high sensitivity and good specificity, such as enzyme immunoassays and immunoblotting. At present, the whole antigen from hydatid cyst fluid (WHCF) is the major source of antigens for the immunodiagnosis of human hydatid disease (Eckert *et al.*, 1984; Guisantes, 1997), the criteria for antigen selection being the presence of antigen 5 (Capron *et al.*, 1967) and/or antigen B (Oriol *et al.*, 1971). For the immunodiagnosis of dog echinococcosis, the antigen more commonly employed is the somatic antigen from sonicated protoscoleces of *Echinococcus granulosus* (Gasser *et al.*, 1989; 1993; Benito *et al.*, 2001). WHCF has been extensively characterized by several authors, such as Capron *et al.* (1967), Oriol *et al.* (1971); Varela-Díaz *et al.* (1974), Lightowlers *et al.* (1989), Maddison *et al.* (1989), Siracusano *et al.* (1991), Leggat and McManus (1994), and Ito *et al.* (1999).

In contrast, fewer characterization studies have been carried out on the excretory-secretory (E-S) antigens from protoscoleces of *Echinococcus*; the most relevant

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investigation in this respect has been done by Gasser *et al.* (1989) and Carmena (2001) in *E. granulosus*, and by Auer *et al.* (1988) in *E. multilocularis*. Therefore, the aim of this study was to describe a culture medium to obtain E-S antigens of *E. granulosus* in an attempt to undertake their biochemical and immunochemical characterization for further assessment of their immunodiagnostic value. Some authors have advocated the use of pepsin for the washing of protoscoleces in order to achieve better results in the isolation of protoscoleces (Smyth and Davies, 1974) or messenger RNA (Shepherd *et al.*, 1991). Thus, a group of pepsin-treated protoscoleces was included in this study in order to evaluate their effect on the collection of E-S antigens.

## 2. Materials and methods

### *2.1. Collection of protoscoleces*

The protoscoleces were obtained from sheep liver and lung hydatid cysts following recommended procedures (Smyth, 1967). After their removal from the cysts the protoscoleces were subject to a cycle of washings through successive sedimentations in phosphate buffer saline (PBS), so that to eliminate the remaining hydatid membranes and fluid. A second washing method based on a 0.05% pepsine-treatment (Sigma, St. Louis, USA) in a Hanks salt balanced solution (Gibco, Paisley, UK) at pH 2.0 over 30 minute-shaking (Smyth and Davies, 1974) was used for one of the batches of protoscoleces in an attempt to show any potential differences between both washing procedures.

### *2.2. Short-term culture media*

Protoscoleces were maintained in a medium consisting of PBS buffer at pH 7.2, 10% dextrose and penicillin-streptomycin (Sigma, St. Louis, USA) at concentrations of 10,000 U/ml and 10 mg/ml, respectively. Volumes of 0.5 ml of protoscoleces and 14.5 ml of medium were dispensed in 50 ml Roux flasks (Sarstedt, Nümbrecht, Germany). Cultures were placed in an incubator at 37 °C, a 5% $CO_2$ atmosphere and a 95% relative humidity.

A total of 12 cultures were performed, with 10 and 2 of them including protoscoleces from liver and lung cysts, respectively. Treatment with 0.05% pepsin was tested in two of the cultures containing protoscoleces from liver cysts.

### *2.3. Collection of excretory-secretory proteins*

The medium was renewed every 8 hours after sedimentation of protoscoleces. At each extraction, a protoscoleces aliquot was taken in order to determine their viability. The mean volume removed was replaced by an equal amount of fresh medium. The whole process was performed under a laminar flow chamber and under sterile conditions.

The extracted medium was concentrated in 5,000-dalton membrane Ultrafree 15 filters (Millipore, Bedford, USA) through centrifugation at 3,000g over 30 minutes. The concentrated medium was stored at - 25° C in the presence of 5 mM EDTA and 2 mM-PMSF final volume, as enzymatic inhibitors. The concentration of excretory-secretory proteins thus obtained was determined by the bicinchoninic acid technique (Sigma, St. Louis, USA).

### *2.4. Assay of protoscoleces viability*

At each renewal of the culture medium, protoscoleces viability was determined by microscopic examination and staining with 0.1%-eosin according to the following criteria: a) vital staining: dead protoscoleces take a reddish colour, while living protoscoleces remain unstained; b) morphological appearance: rounded, well-limited outline, orderly arranged hooks and visible calcareous corpuscles; c) physiological behaviour: contractile movements of the protoscoleces body and activity of flame-cells.

## 3. Results

### *3.1. Viability*

The viability period of cultures ranged from 150 to 260 hours. In all of them, protoscolex percent survival consistently exceeded 85% over the first 48 hours of maintenance. From that time onwards, the number of living protoscoleces decreased progressively until complete culture death. Viability of the two cultures containing lung hydatid protoscoleces was found to be longer than that of the ten cultures of protoscoleces from liver cysts (Fig. 1). On the other hand, pepsin-treated protoscolex cultures showed survival curves slightly lower than those from cultures containing protoscoleces washed with PBS buffer through the serial sedimentation system (Fig. 2).

### *3.2. Protein production*

In the cultures performed, a slight but consistent increase of protein concentration in the culture medium

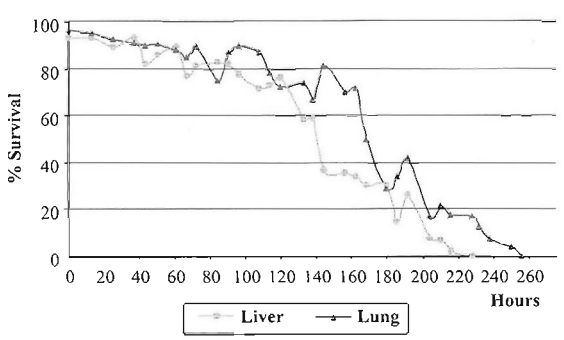


Figure 1. Mean values of survival of protoscoleces in ten cultures from liver hydatid cysts and two cultures from lung hydatid cysts.

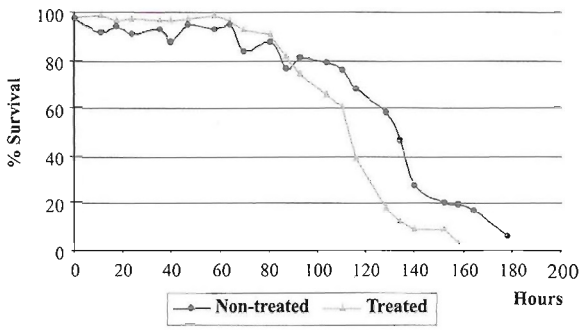


Figure 2. Short term cultures of protoscoleces treated and non-treated with pepsine. Mean values of survival.

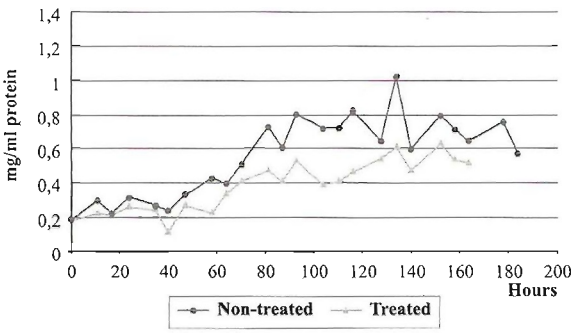


Figure 3. Mean values of protein concentrations (mg/ml) obtained from the cultures of protoscoleces treated and non-treated with pepsine.

was observed over time. While the pattern of protein production was not identical in all the tests, three different phases could be identified:

An early phase comprising the first 50 hours of maintenance, in which the medium protein concentration varied from 0.1 to 0.2 mg/ml. Most of the proteins obtained during this phase are likely to correspond to excretory-secretory products resulting from the parasite metabolism; degradation proteins and cell death would represent a minor component since the percent survival of protoscoleces exceeded 85% throughout the maintenance period.

An intermediate phase, which included the period from the 50$^{th}$ to the 140$^{th}$ hour of culture. During this phase an increase in the medium protein concentration is found probably due to both the excretory-secretory proteins and the proteins released as a result of protoscolex degeneration and lysis.

Phase of death, which would extend from the 140$^{th}$ hour to the end of culture. Protein components during this phase would consist mainly of products resulting from degradation and death of protoscoleces.

No important differences could be found between the amount of proteins produced in cultures of either liver or lung cyst protoscoleces, except during the phase of death when lung cyst protoscoleces yielded greater concentrations of proteins than liver cyst protoscoleces. For pepsin-treated protoscoleces, protein production across all culture phases was lower than that for non-treated protoscoleces (Fig. 3).

## 4. Discussion

In most published studies, cultivation of *Echinococcus* protoscoleces was aimed at either investigating parasite survival at different temperatures (Barriga, 1971; Andersen and Loveless,1978; Casado-Escribano *et al.*, 1986; Casado-Escribano and Rodriguez-Caabeiro, 1987), determining its infectivity (Ohnishi *et al.*, 1984; Casado-Escribano *et al.*, 1992) or analyzing the development of this cestode (Smyth and Davies, 1974). Some of the culture media used by these authors were hydatid fluid, saline, PBS buffer, Hanks balanced solution Parker 199, Parker 858, NCTC 135, CMRL, and RPMI.

The main media used to obtain excretory-secretory antigens of *Echinococcus granulosus* for serodiagnosis of canine echinococcosis were NCTC 135 (Jenkins and Rickard, 1985, 1986) and RPMI (Spinelli *et al.*,1996). However, while a number of studies on the characterization of E-S antigens from *E. multilocularis* has been done (e.g., that by Auer *et al.* [1988] from the maintenance of protoscoleces of this species in RPMI), no such studies are available for *E. granulosus*. To undertake this immunochemical characterization, E-S proteins not contaminated by proteins from the culture medium must be available. The culture medium employed in our study for the short term culture of protoscoleces of *E. granulosus* is free of proteins and other components that may interfere with the material resulting from the parasite metabolism. An easy preparation method and a minimal cost are further advantages of this medium.

As for the initial collection of viable protoscoleces, we observed that their washing through serial sedimentations for removing hydatid fluid and membrane debris is as effective as the washing carried out by pre-treatment with pepsin, a procedure used by other authors to obtain protoscoleces (Smyth and Davies, 1974; Sheperd *et al.*, 1991).

However, pepsin-treated protoscoleces were observed to have a lower survival rate throughout the culture period (Fig. 2), an effect likely caused by enzymatic action on protoscolex protein components, which decreases their vitality and impair their normal metabolic activity. These facts support the choice of washing with PBS buffer through serial sedimentations as the preferred method to obtain protoscoleces for cultivation purposes, since it is less aggressive than purification through enzymatic treatment.

We noted that protoscoleces from lung hydatid cysts had a longer life in cultures than those from liver cysts (Fig. 1). In this regard, our findings differ from those reported by Barriga (1971) who found that the mortality curves for protoscoleces from sheep lung cysts, stored in hydatid fluid and saline at 25 °C were more accelerated than those for liver cysts under the same conditions. A result similar to that of Barriga (1971) was obtained by Casado Escribano *et al.* (1986, 1987) in protoscoleces from sheep and horses hydatid cysts, with the use of PBS buffer, Hanks salt balanced solution, Parker 199 and hydatid fluid, especially at 37 °C.

The optimal period for the production of E-S proteins was found to be the first 48-50 hours of protoscolex maintenance. During this phase, the percentage of viable protoscoleces was higher than 85%, which ensured that most of the protein material present in the maintenance medium would finally consist of excretory-secretory products generated as a result of the parasite metabolic activity. For the same reason, the amount of protein components resulting from cell degradation and lysis in non-viable protoscoleces would be minimal.

No major differences were noted between the amount of protein produced in cultures of liver cyst protoscoleces and those of lung cyst protoscoleces, except during the phase of death, where the latter had higher protein concentrations than those from liver cyst protoscoleces. This is a logical finding since, regardless of the phase, viability is greater for lung cyst protoscoleces; hence, protein production should also be higher.

In pepsin-treated protoscoleces, protein concentration curves in the cultures of enzymatically treated protoscoleces were lower than those produced by non-treated cultures (Fig. 3). This observation seems to be related with the likely decrease of protoscolex vitality as a result of pepsin action, which would also translate into a shorter parasite survival.

In conclusion, we present a simple and low-cost maintenance medium which enables the collection of excretory-secretory proteins of *E. granulosus* protoscoleces in an attempt to achieve their characterization for further use in the immunodiagnosis of echinococcosis/hydatidosis.

## 5. References

Andersen, F.L. and Loveless, R.M. 1978. Survival of protoscolices of *Echinococcus granulosus* at constant temperatures. *J Parasitol,* 64, 78-82.

Auer, H.; Hermentin, K. and Aspöck, H. 1988. Demonstration of a specific *Echinococcus multilocularis* antigen in the supernatant of *in vitro* maintained protoscolices. *Zbl Bakt Hyg*, 268, 416-423.

Barriga, O.O. 1971. Sobrevida de escólices de *Echinococcus granulosus* en solución salina y en líquido hidatídico a diferentes temperaturas. *Boletín Chileno Parasitología,* 26, 80-84.

Benito, A.; Carmena, D.; Spinelli, P.; Martínez, J.; Estibalez, J.J.; Martín de la Cuesta, F. and Guisantes, J.A. 2001. The serological diagnosis of canine echinococcosis by an enzyme immunoassay useful for epidemiological studies. *Res Rev Parasitol*, 61, 17-23.

Capron, A.; Vernes, A. and Biguet, J. 1967. Le diagnostic immuno-électrophorétique de l'hydatidose. In: *Le Kyste Hydatique du Foie*, pp. 27-40. SIMEP, Lyon.

Carmena, D. 2001. Caracterización de antígenos de excreción-secreción de protoescólex de *Echinococcus granulosus* y su aplicación al inmunodiagnóstico de la hidatidosis-equinococosis. Doctoral thesis. University of the Basque County. Vitoria, Spain.

Casado-Escribano, N.; Criado, A.; Jiménez, A.; De Armas, C.; Brasa, C.; Pérez-Serrano, J. and Rodríguez-Caabeiro, F. 1992. Viability of *Echinococcus granulosus* cyst in mice following cultivation *in vitro. Int J Parasitol,* 22, 335-339.

Casado Escribano, N. and Rodríguez-Caabeiro, F. 1987. Estudio de la idoneidad de diferentes medios para el cultivo *in vitro* de *Echinococcus granulosus*. Book of Abstracts from *V Congreso Ibérico de Parasitología*, pp: 367.

Casado Escribano, N.; Rodríguez-Caabeiro, F. and Hernández, S. 1986. *In vitro* survival of *Echinococcus granulosus* protoscolices in several media, at + 4 °C and + 37 °C. *Z Parasitenkd,* 72, 273-278.

Craig, P.S. 1986. Detection of specific circulating antigen, immune complexes and antibodies in human hydatidosis from Turkana (Kenya) and Great Britain, by enzyme-immunoassay. *Parasite Immunol,* 8, 171-188.

Eckert, J.; Gemmel, M.A.; Soulsby, E.J.L. and Matyas, Z. 1984. Guidelines for surveillance, prevention and control of echinococcosis/hydatidosis. *W.H.O.*, Geneva.

Gasser, R.B.; Jenkins, D.J.; Paolillo, E.; Parada, L.; Cabrera, P. and Craig, P.S. 1993. Serum antibodies in canine echinococcosis. *Int J Parasitol*, 23, 579-586.

Gasser, R.B.; Lightowlers, M.W. and Rickard, M.D. 1989. Identification of protein components of *Echinococcus granulosus* protoscolex antigens for specific serodiagnosis of *Echinococcus granulosus* in dogs. *Parasite Immunol,* 11, 279-291.

Guisantes, J.A. 1997. Progress on the laboratory diagnosis of the human hydatid disease. From the recent past till the present. *Archivos Internacionales de Hidatidosis*, 32, 136-140.

Ito, A.; Ma, L.; Schantz, P.M.; Gottstein, B.; Liu, Y.; Chai, J.; Abdel-Hafez, S.K.; Altintas, N.; Joshi, D.D.; Lightowlers, M.W. and Pawlowski, Z.S. 1999. Differential diagnosis for cystic and alveolar echinococcosis using fractions of *Echinococcus granulosus* cyst fluid (antigen B) and *E. multilocularis* protoescolex (EM18). *Am J Trop Med Hyg*, 60, 188-192.

Jenkins, D.J. and Rickard, M.D. 1985. Specific antibody responses to *Taenia hydatigena, Taenia pisiformis* and *Echinococcus granulosus* infection in dogs. *Austr Vet J,* 62, 72-78.

Jenkins, D.J. and Rickard, M.D. 1986. Specific antibody responses in dogs experimentally infected with *Echinococcus granulosus*. *Am J Trop Med Hyg,* 35, 345-349.

Leggatt, G.R. and McManus, D.P. 1994. Identification and diagnostic value of a major antibody epitope on the 12 kDa antigen from *Echinococcus granulosus* (hydatid disease) cyst fluid. *Parasite Immunol,* 16, 87-96.

Lightowlers, M.W., Liu, D., Haralambous, A. and Rickard, M.D. 1989. Subunit composition and specificity of the major cyst fluid antigens of *Echinococcus granulosus*. *Mol Biochem Parasitol*, 37, 171-182.

Maddison, S.E.; Slemenda, S.B.; Schantz, P.M.; Fried, J.A.; Wilson, M. and Tsang, V.C.W. 1989. A specific diagnostic antigen of *Echinococcus granulosus* with an apparent molecular weight of 8 kDa. *Am J Trop Med Hyg,* 40, 377-383.

Ohnishi, K.; Nakao, M. and Inaoka, T. 1984. Viability and infectivity of protoscolices of *Echinococcus multilocularis* stored at different temperatures. *Int J Parasitol* 14, 577-580.

Oriol, R.; Williams, J.F.; Pérez-Esandi, M.V. and Oriol, C. 1971. Purification of lipoprotein antigens of *Echinococcus granulosus* from sheep hydatid fluid. *Am J Trop Med Hyg,* 20, 569-574.

Shepherd, J.C.; Aitken, A. and McManus, D.P. 1991. A protein secreted in vivo by *Echinococcus granulosus* inhibits elastase activity and neutrophil chemotaxis. *Mol Biochem Parasitol,* 44, 81-90.

Siracusano, A.; Ioppolo, S.; Notargiacomo, S.; Ortona, E.; Rigano, R.; Teggi, A.; De Rosa, F. and Vicari, G. 1991. Detection of antibodies against *Echinococcus granulosus* major antigens and their subunits by immunoblotting. *Trans R Soc Trop Med Hyg,* 85, 239-243.

Smyth, J.D. 1967. Studies on tapeworm physiology XI. In vitro cultivation of *Echinococcus granulosus* from the protoescolex to the strobilate stage. *Parasitology,* 57, 111-133.

Smyth, J.D. and Davies, Z. 1974. In vitro culture of the strobilar stage of *Echinococcus granulosus* (sheep strain): a review of basic problems and results. *Int J Parasitol*, 4, 631-644.

Spinelli, P.; Carol, H. and Nieto, A. 1996. Niveles de anticuerpos y antígenos circulantes en perros con infección natural y experimental por *Echinococcus granulosus*. *Inmunología,* 15, 21-29.

Varela-Díaz, V.M.; Coltorti, E.A.; Ricardes, M.I.; Guisantes, J.A. and Yarzábal, L.A. 1974. The immunoelectrophoresis characterization of sheep hydatid cyst fluid antigens. *Am J Trop Med Hyg,* 23, 1092-1096.