

## STRAIN CHARACTERIZATION OF *ECHINOCOCCUS GRANULOSUS* PROTOSCOLECES OF CATTLE ORIGIN USING THE *IN VITRO* VESICULAR DEVELOPMENT

ELISSONDO M.C.\*\*\*, DOPCHIZ M.C.\*\*\*, ZANINI F.\*\*\*, PÉREZ H.\*\*\*, BRASESCO M.\* & DENEGRI G.\*\*\*

### Summary:

The aim of this work was to characterize the strain of protoscoleces of *E. granulosus* of cattle origin using the *in vitro* vesicular development. The *in vitro* development of these samples was compared to samples of sheep origin determined previously by genetic analyses as common sheep strain (G1). There were similarities between sheep and cattle samples not only in the time of microcysts formation, but also in the development process. 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. In the sheep samples, microcysts were observed between 19 and 20 days. In the cattle samples, microcysts appeared between 20 and 23 days. The coincidence between the development times and physiological characteristics found in the present study may indicate that the parasites from cattle and sheep were of the same strain.

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

**Résumé :** CARACTÉRISATION DE SOUCHES DE PROTOSCOLEX D'*ECHINOCOCCUS GRANULOSUS* D'ORIGINE BOVINE PAR LA TECHNIQUE DE CULTURE *IN VITRO*

L'objectif de notre étude était de caractériser des souches de protoscolex d'*E. granulosus* d'origine bovine par la technique de la culture *in vitro*. Le développement *in vitro* des protoscolex a été comparé avec celui des protoscolex d'origine ovine déterminée auparavant par des analyses génétiques. On a observé des similitudes entre les protoscolex d'origines ovine et bovine, non seulement dans le temps de formation des microkystes, mais aussi dans le processus de développement. Des protoscolex vésiculeux et des protoscolex avec une vésicule postérieure sont apparus pendant la première semaine d'incubation. Après 14 jours de culture, est apparue une fine membrane laminée à l'une des extrémités des protoscolex. Avec les protoscolex d'origine ovines, on a observé des microkystes entre les 19<sup>ème</sup> et 20<sup>ème</sup> jours; avec ceux d'origine bovines, entre les 20<sup>ème</sup> et le 23<sup>ème</sup> jours. Les coïncidences concernant les temps de développement et les caractéristiques physiologiques observées au cours de cette étude permettent de penser que les parasites d'origines bovine et ovine étaient de même souche.

**MOTS CLÉS :** *Echinococcus granulosus*, hydatidose, protoscolex, culture *in vitro*, souche.

## INTRODUCTION

Among the parasitic helminths, *Echinococcus granulosus* is one of the common and cosmopolitan tapeworms; it produces extremely severe clinical forms of disease (Matossian *et al.*, 1977). Its life cycle has two stages, *i.e.*, an adult stage during which the parasite inhabits the intestine of canids and sheds its eggs into the environment and a phase with

hydatid cysts in the tissues of herbivores and humans (Fiori *et al.*, 1988).

*In vitro* culture of *E. granulosus* protoscoleces and their subsequent development into cysts or strobilar stages has been the subject of study by a number of investigators. The first successful *in vitro* cultivation in a cystic direction was reported by Smyth (1962, 1967). Since then, a significant progress was made using a variety of media and conditions (Benex, 1968; Brudnjak *et al.*, 1970; Heath & Osborn, 1976; Casado *et al.*, 1986; Rogan & Richards, 1986; Rodriguez-Caabeiro & Casado, 1988; Casado & Rodriguez-Caabeiro, 1988a, b, 1989; Denegri *et al.*, 2002).

However, *E. granulosus* shows great intraspecific variability. This variability was brought to light by Smyth & Davies (1974a), who observed differences in the *in vitro* strobilar development between protoscoleces from ovine and equine cysts. Similar results have been obtained with samples from other hosts and/or different geographical origin, using several analytical techniques related to parasite biochemistry, genetics, morphology, immunology, etc. (Thompson & Allsopp, 1988;

\* Laboratorio de Zoonosis Parasitarias, Dpto. de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Argentina.

\*\* Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

\*\*\* Programa de Control de Hidatidosis y Zoonosis de Tierra del Fuego, Piedra Buena 349, (9420) Río Grande, Tierra del Fuego, Argentina.

Correspondence: Dr Guillermo Denegri, Laboratorio de Zoonosis Parasitarias, Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250 (7600) Mar del Plata, Buenos Aires, Argentina.

Tel.: (54) (223) 4752426 – Fax: (54) (223) 4753150.

E-mail: [gdenegri@mdp.edu.ar](mailto:gdenegri@mdp.edu.ar)

Thompson, 1995). The parasite occurs as a series of genetic variants or strains, which differ in a wide variety of criteria that impact on the epidemiology, pathology, and control of cystic hydatid disease (Thompson & Lymbery, 1988; Thompson, 1995). To date, 10 distinct genotypes have been identified within *E. granulosus*. Eight of these genotypes were identified as: common sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7), and cervid strain (G8). The G9 genotype was detected in human cystic hydatid cases from Poland (Bowles *et al.*, 1992). A novel genotype named the Fennoscandian cervid strain (G10) was characterized by Laviakinen *et al.* (2003).

Five different strains occur in Argentina: G1, G2, G5, G6 and G7 (Rosenzvit *et al.*, 1999). In Buenos Aires Province, located in the Pampas region, only genotypes G1, G6 and G7 were found in isolates of different hosts. Moreover, in cattle isolates only G1 strain was identified (Kamenetzky *et al.*, 2002; Rosenzvit *et al.*, 2002).

Ponce-Gordo & Cuesta-Bandera (1997) compared different strains of *E. granulosus* using the *in vitro* vesicular culture technique. They found differences not only in the time of microcysts formation, but also in the development process. They concluded that, at least in relation to the initial stages of development to cyst (in vitro, microcyst formation), the three Spanish strains of *E. granulosus* show clear and differential physiological characteristics.

The aim of the present work was to characterize the strain of protoscoleces of *E. granulosus* of cattle origin using the *in vitro* vesicular development.

## MATERIALS AND METHODS

Hydatid cysts from naturally infected bovine lungs and livers were obtained from two abattoirs situated in the southeast of Buenos Aires Province, Argentina. The *in vitro* development of these samples was compared to samples of sheep origin determined previously using a polymerase chain reaction (PCR)-linked restriction fragment length polymorphism (RFLP) method (Bowles & McManus, 1993) as common sheep strain (G1) (Cerrone *et al.*, 1999; Cerrone & Targovnik, 2002). Ovine cysts were provided by the Hydatid Control Program of Tierra del Fuego Province, Patagonian region.

Prior to arrival in the laboratory, all samples were maintained as intact cysts at 5-10°C in appropriate containers for several hours to a maximum of two days.

Each cyst was considered as an individual sample. Processing of all samples was carried out following the protocol described by Smyth & Davies (1974b). Pro-

toscoleces were removed from cysts by aseptic techniques and washed several times with PBS. Viability was assessed using the methylene blue exclusion test (Casado *et al.*, 1986). Samples with vitality percentages less than 60 % were discarded.

Once a sample was accepted, aliquots of 1,500 protoscoleces were transferred to Leighton tubes, one to five replicates per sample, depending on quantity of available protoscoleces. The culture medium was 199 (Gibco) supplemented with 20 % (v/v) fetal calf serum, 100 IU penicillin, 100 µg/ml streptomycin, and 4 mg/ml glucose, at pH 7.2-7.4. The pH was monitored during the culture period by means of the pH indicator (phenol red) incorporated in the 199 medium. Cultures were maintained at 37°C and the medium was changed every three-four days. A total of 12 samples (10 bovine, two ovine) were cultured.

Development was followed microscopically every day. Development time until microcysts was determined in days. Due to biological variability, protoscoleces from the same sample develop at different rates. The data recorded for each sample correspond to the moment when a stage was reached by the most advanced individuals of the culture.

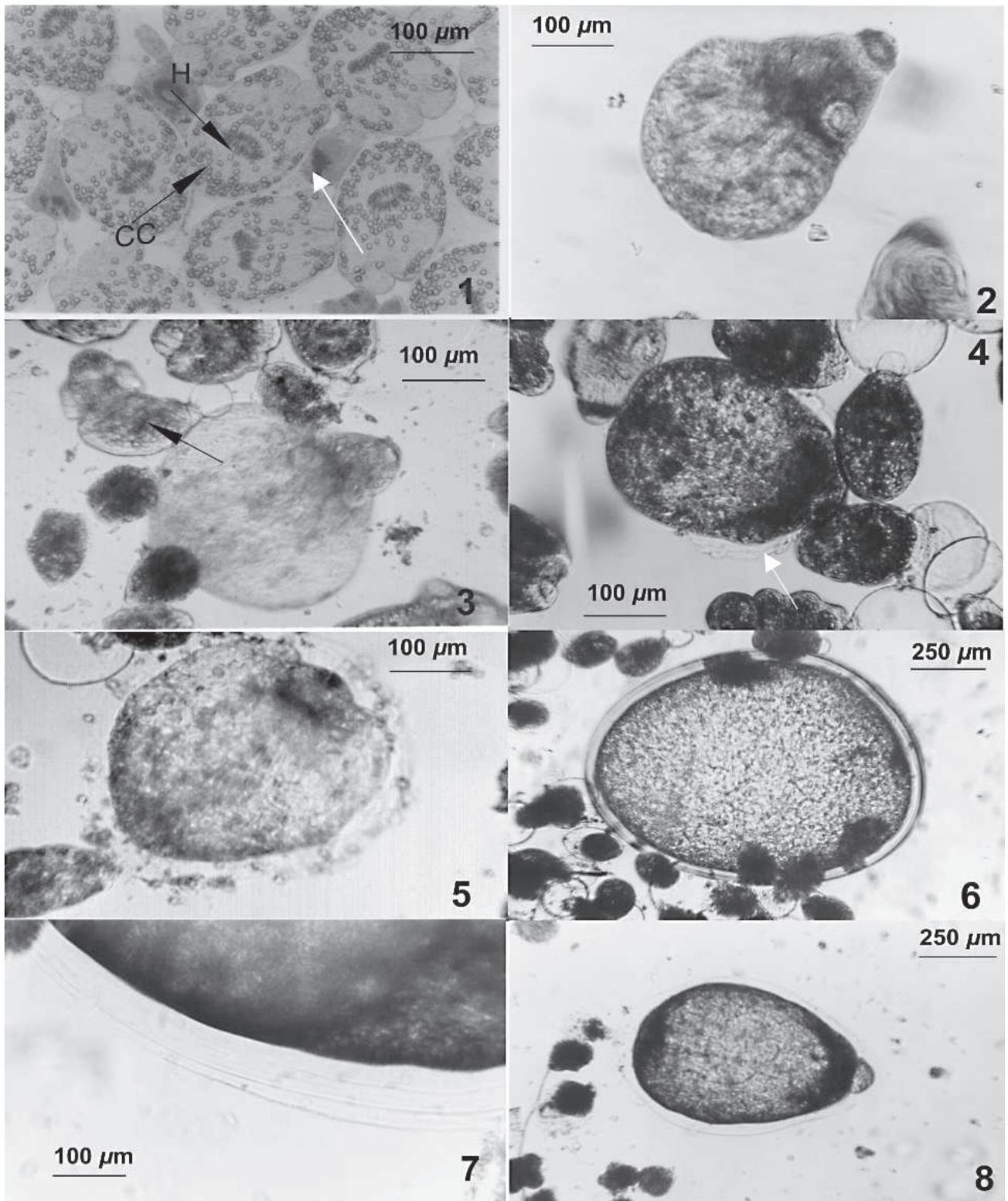
## RESULTS

There were similarities between sheep and cattle samples not only in the time of microcyst formation, but also in the development process. At the beginning of the culture, in both sheep and cattle samples, protoscoleces were invaginated (Fig. 1). After 48 to 72 hours of incubation some evaginated protoscoleces could be observed. The number was variable in the different cultures, but it never reached 100 %.

Vesiculated protoscoleces (Figs 2, 3) and protoscoleces with posterior bladders (Fig. 3) appeared during the first week of incubation. Moreover, a considerable increment in the size could be observed. After 14 days of culture a laminated layer appeared like a fine membrane in one of the extremes of the protoscoleces (Fig. 4).

All ovine samples and the 70 % of bovine samples reached the microcyst formation. In cattle samples microcysts appeared between 20 and 23 days. Some samples showed microcysts formation between 24-38 days (Figs 5, 6). In sheep samples microcysts were observed between 19 and 20 days, occasionally later, in 21 to 27 days (Figs 7, 8).

Table I shows a comparison between previous studies on *in vitro* vesicular development of *E. granulosus* using sheep samples and the results obtained in the present study working with protoscoleces of sheep and cattle origin.



Figs 1-8. – *In vitro* development of protoscoleces of *Echinococcus granulosus* of cattle and sheep origin: **1.** Protoscoleces obtained of lung cattle cysts. The typical shape of protoscoleces is observed, hooks (H) and calcareous corpuscles (CC). Dead stained protoscolex (white arrow). Methylene blue exclusion test 1:10.000. **2.** Vesiculated protoscolex of sheep origin (12 days of culture). **3.** Vesiculated protoscolex of cattle origin (10 days of culture). Note the increment in size. An evaginated protoscolex showing a posterior bladder is observed (arrow). **4.** Vesiculated protoscoleces of sheep origin (14 days of culture) showing the appearance of laminar layer (white arrow). **5.** Microcyst of cattle origin showing laminated layer (20 days of culture). **6.** Microcyst of cattle origin completely developed (48 days of culture). **7.** Microcyst of sheep origin. Detail of the laminated membrane (50 days of culture). **8.** Microcyst of sheep origin (50 days of culture).

| Author                              | Geographical origin of samples              | Hosts of procedence | Composition of culture medium | Beginning of vascularization (in days) | Appearance of laminar layer (in days) | Appearance of miniature cysts (in days) | Obtained cyst (%) | Maximum survival (in days) |
|-------------------------------------|---------------------------------------------|---------------------|-------------------------------|----------------------------------------|---------------------------------------|-----------------------------------------|-------------------|----------------------------|
| Own data                            | Southeast Buenos Aires Province (Argentina) | Cattle              | Medium 199 + serum            | 5                                      | 14                                    | 20-38                                   | 1.6               | 95                         |
| Own data                            | Tierra del Fuego (Patagonia, Argentina)     | Sheep               | Medium 199 + serum            | 7                                      | 14                                    | 19-27                                   | 3                 | 119                        |
| Rodríguez-Caabeiro (1988)           | Spain                                       | Sheep               | Medium 199 + serum            | 6                                      | 9                                     | 15                                      | 1                 | 90                         |
| Ponce-Gordo & Cuesta-Bandera (1997) | Spain                                       | Sheep               | CMRL + serum                  | 2-4                                    | 12-17                                 | 19-37                                   | 1-10              | Not indicated              |

Table I. – Comparison of present and previous works on *in vitro* vesicular development of protoscoleces of *Echinococcus granulosus* of sheep and cattle origin.

## DISCUSSION

The comparison between the cultures with ovine and bovine material showed that there are no differences in the development process and in the time of microcyst formation. During the first week vesicular protoscoleces are obtained. The formation of the laminated layer began in the second week. Microcysts were obtained approximately after 20 days of culture. The times of microcyst formation found in the sheep samples are within range of published data (Table I).

Ponce-Gordo & Cuesta-Bandera (1997) worked with cysts of sheep, cattle, horses, pigs and humans. This was the first study where pig, cattle and humans samples have been analysed using the *in vitro* vesicular technique. 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.

Our results with cattle samples are clearly different from those indicated by Ponce-Gordo & Cuesta-Bandera (1997). Working with this material microcyst formation was achieved in 20-38 days (Elissondo *et al.*, 2004).

Interestingly, the proved viability in *in vitro* culture of cattle samples have been corroborated in *in vivo* cultivation in mice as well as in strobilar development in experimentally infected dogs (Dopchiz *et al.*, 2002). Moreover, the sheep strain (G1 genotype) was determined in hydatid cysts obtained from cattle slaughtered in abattoirs of the study area (Dopchiz *et al.*, 2002). This concur with the results obtained in genetic analyses by Rosenzvit *et al.* (1999, 2002) and Kamenetzky *et al.* (2002): only sheep strain (G1) was identified in

cattle isolates of Buenos Aires Province. The material used for the *in vitro* culture in the present study was recently characterized by molecular techniques to determine the involved strains and sheep strain (group G1/G2) was determined (unpublished data).

Ponce-Gordo & Cuesta-Bandera (1997) characterized the Spanish strains using the *in vitro* vesicular development and found differential physiological characteristics working with samples of different animal origin (sheep, pig and horse). They concluded that their results show the validity of the *in vitro* vesicular culture technique for strain identification and characterization in *E. granulosus*. Besides, their results concur with results obtained in previous genetic analyses (Cuesta-Bandera, 1988; Cuesta-Bandera *et al.*, 1988; Siles-Lucas *et al.*, 1993, 1994, 1996).

The coincidence between the development times and physiological characteristics found in the present study working with samples of sheep and cattle origin indicate that the strain is the same: the common sheep strain. Taking this and the molecular analyses into account, we consider the utility of using the *in vitro* vesicular development as an alternative technique to characterize the strains of *E. granulosus*.

## ACKNOWLEDGEMENTS

The authors thank Dr González, Dr Tessi, Dr Viglietti and Sr Chasma for their contributions and Lidia Formaini for providing linguistic assistance. This work was supported by the PICT 02. N° 01-12535, préstamo BID 1201/OC-AR. Partial financial support of this work was provided by Fundación Alberto J. Roemmers.

## REFERENCES

- BENEX J. Considérations expérimentales nouvelles sur l'évolution *in vitro* en milieu liquide des larves d'*Echinococcus granulosus*. *Annales de Parasitologie Humaine et Comparée*, 1968, 43, 561-572.
- BOWLES J. & MCMANUS D.P. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. *Molecular and Biochemical Parasitology*, 1993, 57, 231-240.
- BOWLES J., BLAIR D. & MCMANUS D.P. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology*, 1992, 54, 165-173.
- BRUDNJAK Z., CVETNIC S. & WILKERHAUSER T. Cystic development of the protoscoleces and brood capsules of *Echinococcus granulosus* in cell cultures and cell-free media. *Veterinarski Arhiv (Zagreb)*, 1970, 40, 292-296.
- CASADO N. & RODRIGUEZ-CAABEIRO F. Cultivo *in vitro* de protoescolex de *Echinococcus granulosus* en dirección vesicular. I. Efecto del pH. *Revista Ibérica de Parasitología*, 1988a, 48, 17-23.
- CASADO N. & RODRIGUEZ-CAABEIRO F. Cultivo *in vitro* de protoescolex de *Echinococcus granulosus* en dirección vesicular. II. Estudios de la idoneidad de diferentes medios. *Revista Ibérica de Parasitología*, 1988b, 48, 155-163.
- CASADO N. & RODRIGUEZ-CAABEIRO F. Ultrastructural study of *in vitro* larval development of *Echinococcus granulosus* protoscoleces. *International Journal of Parasitology*, 1989, 19, 21-28.
- CASADO N., RODRIGUEZ-CAABEIRO F. & HERNÁNDEZ S. *In vitro* survival of *Echinococcus granulosus* protoescolices in several media, at 42° C and 37° C. *Zeitschrift für Parasitenkunde*, 1986, 72, 273-278.
- CERRONE G. & TARGOVNIK H.M. Análisis del genoma de *Echinococcus granulosus*, in: Situación de la hidatidosis-echinococcosis en la República Argentina, Denegri G., Elissondo M.C. & Dopchiz M.C. (eds). Martín, Mar del Plata, Argentina, 2002, p. 41-48.
- CERRONE G., FERNANDEZ E., FERRARA E., DALLA FONTANA L., ZANINI F., JENSEN O. & TARGOVNIK H.M. *Echinococcus granulosus* strain situation in Argentina. *Archivos Internacionales de la Hidatidosis*, 1999, 33, 327.
- CUESTA-BANDERA C. Especiación, epidemiología y control de la hidatidosis en España. Proceedings of VIII Reunión Científica Nacional de la Asociación Española de Hidatidología (Córdoba, Spain), 1988, p. 38-42.
- CUESTA-BANDERA C., MCMANUS D.P. & RISHI A.K. Characterization of *Echinococcus granulosus* of Spanish origin by DNA restriction endonuclease analysis and Southern blot hybridization. *International Journal of Parasitology*, 1988, 18, 137-141.
- DENEGRI G., ELISSONDO M.C. & DOPCHIZ M.C. Impacto de los modelos experimentales de cultivo *in vitro* e *in vivo* para el tratamiento de la hidatidosis, in: Situación de la hidatidosis-echinococcosis en la República Argentina, Denegri G., Elissondo M.C., & Dopchiz M.C. (eds). Martín, Mar del Plata, Argentina, 2002, p. 197-203.
- DOPCHIZ M.C., ELISSONDO M.C. & DENEGRI G. Consideraciones epidemiológicas de la hidatidosis-echinococcosis en el sudeste de la provincia de Buenos Aires, in: Situación de la hidatidosis-echinococcosis en la República Argentina, Denegri G., Elissondo M.C., & Dopchiz M.C. (eds). Martín, Mar del Plata, Argentina, 2002, p. 65-75.
- ELISSONDO M.C., DOPCHIZ M.C., BRASESCO M. & DENEGRI G. *Echinococcus granulosus*: First report of microcysts formation from protoscoleces of cattle origin using the *in vitro* vesicular culture technique. *Parasite*, 2004, 11, 415-418.
- FIORI P.L., MONACO G., SCAPPATICCI S., PUGLIESE A., CANU N. & CAPPUCCELLINI P. Establishment of cell cultures from hydatid cysts of *Echinococcus granulosus*. *International Journal of Parasitology*, 1988, 18, 297-305.
- HEATH D.D. & OSBORN P.J. Formation of *Echinococcus granulosus* laminated membrane in a defined medium. *International Journal of Parasitology*, 1976, 6, 467-471.
- KAMENETZKY L., GUTIERREZ A.M., CANOVA S.G., HAAG K.L., GUARNERA E.A., PARRA A., GARCÍA G.E. & ROSENZVIT M.C. Several strains of *Echinococcus granulosus* infect livestock and humans in Argentina. *Infection, Genetics and Evolution*, 2002, 2, 129-136.
- LAVIAKINEN A., LEHTINEN M.J., MERI T., HIRLEVA-KOSKI V., & MERI S. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. *Parasitology*, 2003, 127, 207-215.
- MATOSSIAN M.R., RICKARD M.D. & SMYTH J.D. Hydatidosis: a global problem of increasing importance. *Bulletin of the World Health Organization*, 1977, 55, 499-507.
- PONCE-GORDO E. & CUESTA-BANDERA C. *Echinococcus granulosus*: characterization of the Spanish strains using *in vitro* vesicular development. *Journal of Helminthology*, 1997, 71, 61-67.
- RODRÍGUEZ-CAABEIRO F. & CASADO N. Evidence of *in vitro* germinal layer development in *Echinococcus granulosus* cysts. *Parasitology Research*, 1988, 74, 558-562.
- ROGAN M.T. & RICHARDS K.S. *In vitro* development of hydatid cysts from posterior bladders and ruptured brood capsules of equine *Echinococcus granulosus*. *Parasitology*, 1986, 92, 379-390.
- ROSENZVIT M.C., ZHANG L.H., KAMENETZKY L., CANOVA S.G., GUARNERA E.A. & MCMANUS P. Genetic variation and epidemiology of *Echinococcus granulosus* in Argentina. *Parasitology*, 1999, 118, 523-530.
- ROSENZVIT M.C., GUTIERREZ A. & KAMENETZKY L. Variación genética en *Echinococcus granulosus*, in: Situación de la hidatidosis-echinococcosis en la República Argentina, Denegri G., Elissondo M.C. & Dopchiz M.C. (eds). Martín, Mar del Plata, Argentina, 2002, p. 31-40.
- SILES-LUCAS M., CUESTA-BANDERA C. & CESAR-BENITO M. Random amplified polymorphic DNA technique for speciation studies of *Echinococcus granulosus*. *Parasitology Research*, 1993, 79, 343-345.
- SILES-LUCAS M., FELLEISEN R., CUESTA-BANDERA C., GOTTSTEIN B. & ECKERT J. Comparative genetic analysis of Swiss and Spanish isolates of *Echinococcus granulosus* by southern hybridization and random amplified polymorphic DNA technique. *Applied Parasitology*, 1994, 35, 107-117.

- SILES-LUCAS M., BENITO M.C. & CUESTA-BANDERA C. *Echinococcus granulosus*: genomic and isoenzymatic study of Spanish strains isolated from different intermediate hosts. *Veterinary Parasitology*, 1996, *63*, 273-282.
- SMYTH J.D. Studies on tapeworm physiology. X. Axenic cultivation of the hydatid organism *Echinococcus granulosus*: establishment of a basic technique. *Parasitology*, 1962, *52*, 441-457.
- SMYTH J.D. Studies on tapeworm physiology. XI. *In vitro* cultivation of *Echinococcus granulosus* from the protoscolex to the strobilate stage. *Parasitology*, 1967, *57*, 111-133.
- SMYTH J.D. & DAVIES Z. Occurrence of physiological strains of *Echinococcus granulosus* demonstrated by *in vitro* culture of protoescoleces from sheep and horse hydatid cysts. *International Journal for Parasitology*, 1974a, *4*, 443-445.
- SMYTH J.D. & DAVIES Z. *In vitro* culture of the strobilar stage of *Echinococcus granulosus* (sheep strain): a review of basic problems and results. *International Journal for Parasitology*, 1974b, *4*, 631-644.
- THOMPSON R.C.A. Biology and systematics of *Echinococcus*, in: *Echinococcus* and hydatid disease, Thompson R.C.A. & Lymbery A.J. (eds). CAB International, Wallingford, Oxon, U.K., 1995, p. 1-50.
- THOMPSON R.C.A. & ALLSOPP C.E. Hydatidosis: veterinary perspectives and annotated bibliography. CAB International, Wallingford, UK, 1988, 246 p.
- THOMPSON R.C.A. & LYMBERG A.J. The nature, extent and significance of variation within the genus *Echinococcus*. *Advances in Parasitology*, 1988, *27*, 209-259.

Reçu le 7 janvier 2005  
 Accepté le 2 mars 2005