Available online at www.sciencedirect.com
SCIENCE DIRECT
Veterinary Parasitology 133 (2005) 71–77
veterinary parasitology
www.elsevier.com/locate/vetpar

Viability and infectiousness of eggs of *Echinococcus granulosus* aged under natural conditions of inferior arid climate

Paula Sánchez Thevenet [a,*], Oscar Jensen [b], Ricardo Drut [c], Gloria E. Cerrone [d], María S. Grenóvero [e], Héctor M. Alvarez [a], Héctor M. Targovnik [d], Juan A. Basualdo [f]

[a] *Departamento de Bioquímica, School of Natural Science, Facultad de Ciencias Naturales, Universidad Nacional de la Patagonia San Juan Bosco, Piso 2 Ciudad Universitaria Km. 4, Comodoro Rivadavia, Chubut 9000, Argentina*
[b] *Hydatidosis Control Program, Province of Chubut, Argentina*
[c] *Pathology Unit, Sor María Ludovica Children's Hospital, La Plata, Argentina*
[d] *Genetics and Molecular Biology Department, School of Pharmacy and Biochemistry, Universidad de Buenos Aires, Argentina*
[e] *Biostatisctics Institute, School of Health Sciences, Universidad Adventista del Plata, Argentina*
[f] *Microbiology and Parasitology, School of Medical Sciences, Universidad Nacional de La Plata, Argentina*

Received 2 April 2005; received in revised form 13 May 2005; accepted 18 May 2005

Abstract

We studied the viability and infectiousness of aged *Echinococcus granulosus* eggs by in vivo evaluation in ovines. Our results demonstrated that after 41 months of ageing of the eggs under environmental conditions of an inferior arid climate (Patagonia, Argentina), they were still able to produce infection in 4/4 ovines challenged with 1200 eggs per ovine. In the ovines experimentally infected with these aged eggs, the occurrence of hepatic and pulmonary cysts was determined by necropsy, histologic and genetic studies. The eggs were found in a semi-senescent stage, thus keeping their capacity to generate an infection in the intermediary ovine host.

Keywords: *Echinococcus granulosus*; Eggs; Viability; Environment; Patagonia, Argentina

1. Introduction

Other studies on survival of eggs of taeniids in natural environmental conditions (Coman, 1975; Gemmel et al., 1987; Ilsoe et al., 1990; Wachira et al., 1991; Veit et al., 1995) reported a 1 year follow-up maximum.

* Corresponding author. Tel.: +54 297 4550339.; fax: +54 297 4550339.
E-mail address: psanchez@unpata.edu.ar (P.S. Thevenet).

0304-4017/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2005.05.048

However, our previous studies demonstrated that the time of permanence of eggs of *Echinococcus granulosus* in canine feces in an inferior arid climate condition has reached 41 months (Sánchez Thevenet et al., 2003).

Silverman (1956) proposed that the ability of taeniid eggs to produce experimental infection in an appropriate intermediary host (IH) depends, among other factors, on the maturity stage of the eggs. Gemmel et al. (1987) reported that the maturing and ageing processes of taeniid eggs continue in the environment. Eggs from *Taenia hydatigena* were observed to undergo different stages, namely: immature, mature, semi-senescent and dead (Gemmel et al., 1987). For *Taenia pisiformis*, Coman and Rickard (1977) described the senescent stage of eggs aged for 9 days at 36–38 °C and 87–93% relative humidity as characterized by the eggs producing evidence of initial infection in rabbits but not completing their development to cysticercus.

The viability of taeniid eggs can be evaluated by means of in vitro and in vivo tests (Coman and Rickard, 1977). For fresh eggs of *E. granulosus*, the infectiousness evaluated in vivo persists in the absence of positive activity in the viability in vitro tests (Coman, 1975).

The viability of aged eggs of *E. granulosus* and their capacity to produce infection in an intermediary host has not been reported yet in studies of over one year follow-up.

The objective of the present study was to determine the characteristics of viability and infectiousness of eggs of *E. granulosus*, aged in natural conditions of an inferior arid climate for 41 months in Patagonia, Argentina.

For this study we used *E. granulosus* eggs, which previously proved to be infectious and produced viable hydatid cysts in all ten challenged sheep when they were fresh (Lightowlers et al., 1999).

2. Materials and methods

An experimental, longitudinal, prospective and individual type of design, with control group, was applied.

2.1. Aged eggs of E. granulosus

Eggs from mature *E. granulosus* used in the confirmation trial for the effectiveness of the EG95 vaccine (Lightowlers et al., 1999) were used. The eggs remained outdoors placed on the original canine faeces deposited onto 1 m^2 surface stratum of soil for 41 months and exposed to the natural environmental conditions between October 1996 and February 2000, on the experimental field of the Hydatidosis Control Program, in Sarmiento, Province of Chubut, Argentina (45.30°S, 69.28°W) (Sánchez Thevenet et al., 2003). After the exposure period, the eggs were recovered following the conventional flotation technique of Sweatman and Williams (1963) to obtain an eggs suspension in drinking water. The suspension had a concentration equal to 4 eggs/ml, determined by triplicate counts in Neubauer's chamber.

2.2. Viability and infectiousness trial

The viability and infectiousness of the eggs of *E. granulosus* naturally aged were evaluated in vivo (Coman, 1975; Coman and Rickard, 1977; Ilsoe et al., 1990; Veit et al., 1995) in a clinical essay by causing the experimental infection of ovines.

Six 7-month-old ovines of the Texel breed, from a hydatidosis-free area, handed over by the Corporación de Fomento de la Provincia de Chubut (CORFO), were used. The lack of infection was proved by means of serology prior to inoculation, carried out with the K-Elisa test according to Ibrahem et al. (1996) and Kittelberger et al. (2002), by using total hydatid liquid and antigen B of *E. granulosus* as antigens.

Two out of the six ovines were used as negative controls (ovines 1 and 2) and the other four were exposed to the infection (ovines 3–6). Ovines 3–6 were inoculated and 300 ml of the suspension of the aged eggs of *E. granulosus* in drinking water was applied per o.s. to them, according to Sweatman and Williams (1963). The control ovines were given 300 ml of drinking water. Before inoculation, all ovines were kept in fasting and continuous water supply for 24 h. The ovines were hand-reared entirely in a restricted access area of the experimental field under stringent conditions, grazed egg-free pasture and drinked uncontaminated water.

2.3. Presence of cysts

A post-mortem search of hydatid cysts (HC) was performed in the exposed ovines and in the controls,

according to the methodology of Gemmel et al., 1987; Cabrera et al., 1995; Lightowlers et al., 1999; Kittelberger et al., 2002. Following the protocol of Lightowlers et al. (1999), all animals were inspected 13 months post-challenge. The carcasses and omentum were examined superficially. The livers, spleens, hearts, brains and kidneys were examined by section at 2 mm intervals. The lungs were dissected at 6 mm intervals, with a palpation search to detect the presence of cysts. The number, location and size of cysts found were recorded. The cysts were examined macroscopically, and classified as viable if they had a fluid, crystalline liquid inside a cavity (Lightowlers et al., 1999). The fertility of the cysts was determined by the presence of protoscoleces. The cysts with no protoescoleces were considered sterile. (Cabrera et al., 1995).

2.4. Corroborating studies

Samples of the cystic material found in exposed ovines and surrounding tissue were fixed in formaldehyde 10% for histologic study performed by inclusion in paraffin and Hematoxylin–Eosin stain (Coman and Rickard, 1977). The pulmonary and hepatic tissue samples from control ovine 1 were also fixed in formaldehyde for histologic study.

The pulmonary cysts found were kept in alcohol 70% for studies of identification of a specific sequence of *E. granulosus*. The genomic DNA was purified by conventional methods (Maniatis et al., 1989). The obtained DNA was concentrated and purified with Microcon-100 columns, following the manufacturer's indications. Then, the PCR amplification of specific sequences was carried out by using the primers: EgD, 5′-GCACAAAATACTGAAGACGAC-3′ and EgR, 5′-AATCTTCGGCTTCACAACTG-3′ previously designed from the oEg01 sequence (Cerrone and Targovnik, 2002; GenBank™, Genome Survey Sequences Division, Accession number: BH753872). Then 200 ng of genomic DNA isolated from the *E. granulosus* cysts were amplified and, to control the reaction, purified DNA of ovine hydatid cysts from Australia (kindly handed over by Dr. Marshall Lightowlers) were used. The reaction was performed in 100 μl of final volume containing buffer 1× (50 mM KCl, 20 mM Tris–HCl, pH 8.4), 2.5 mM $MgCl_2$, 6% DMSO, 200 μl of each dNTP (dATP, dCTP, dTTP and dGTP), 50 pmol of each direct and reverse (GIBCO BRL) and two units of DNA polymerase of Thermus acquaticus (GIBCO, BRL). The PCR reaction was done in a PTC 100 Thermocycler (MJ Research Inc.) with an initial DNA denaturalization at 94 °C for 5 min and 30 amplification cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. After the last cycle, the samples were incubated for 5 min at 72 °C to secure the final extension. The specific product of 165 pb (10 μl) was observed in an agarose gel at 2%/TBE (Tris–borate/EDTA) in a UV transluminator after ethidium bromide stain and photographed.

2.5. Environmental conditions

The eggs were exposed during 41 months to an inferior arid climate that is characterized by great thermal amplitude, with warm summers and cold winters with frequent frosts (Unesco, 1979). The weather data were obtained from the Instituto Nacional de Tecnología Agropecuaria (INTA) station, in Sarmiento, Chubut province. The temperature range was 37 to −3 °C, with a mean annual temperature of 10 °C. There were also average winds and low level of precipitation, under 300 mm/year.

2.6. Descriptive statistic techniques

Frequency distribution and summary measures were used to evaluate differences between the numbers of cysts found according to their location. We considered values of $P < 0.05$ to be significant.

2.7. Biosafety regulations and ethical aspects

In all instances of the study, the biosafety regulations and the national and international regulations referring experimental studies on animals were respected (Eckert et al., 2001).

3. Results and discussion

The four ovines challenged with eggs of *E. granulosus* naturally aged showed cysts (Fig. 1) while no such forms were found in the control ovines. The total of cysts found in the four ovines challenged was

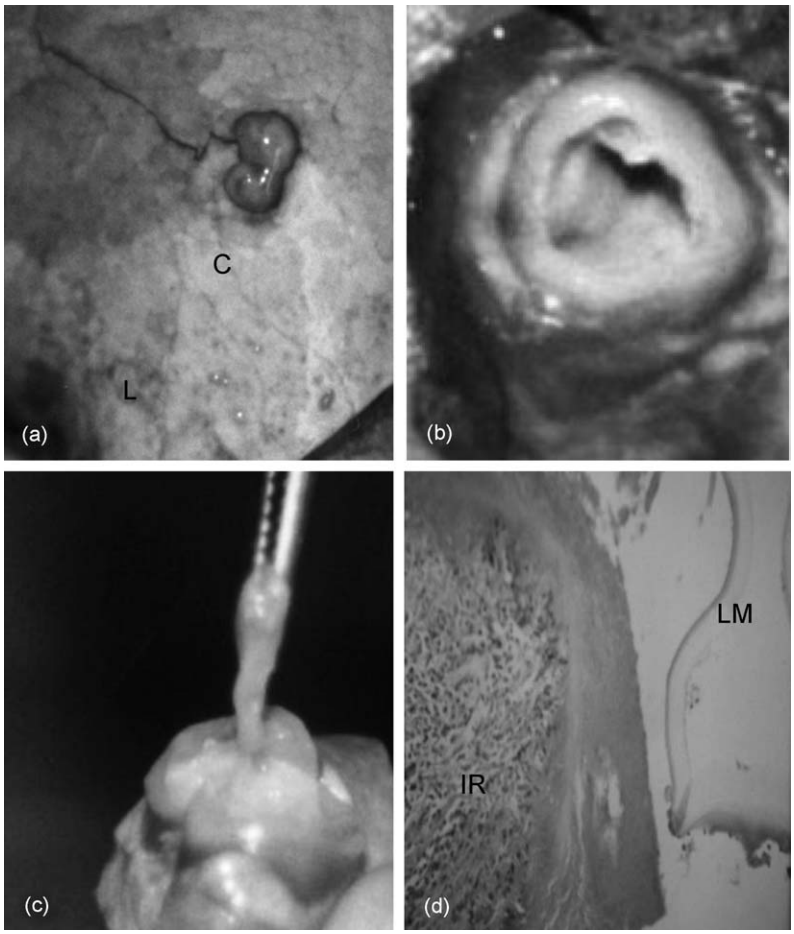

Fig. 1. (a) Cyst (C) in lung (L) developed from aged eggs of *Echinococcus granulosus*; (b) central cavity of pulmonary cyst; (c) internal membrane of pulmonary cyst; (d) laminar membrane (LM), with intense pericystic lympho-immunoblastic and histiocite reaction (IR). Pulmonary cyst in ovine 4. Hematoxilina–Eosina 10×.

16, with a representative value of 4 ± 2 cysts/ovine. The location and number of cysts detected per ovine is shown in Table 1. As much as 63% of the cysts were found in the liver, and 37% in the lung. There were more cyst in the liver although it was not statistically significant. The cysts turned out to be unilocular, except for one ovine whose cysts were bilocular. The average diameter of cysts was 6.5 ± 2.6 mm (range = 5–11). Six cysts were selected randomly, five pulmonary and one hepatic, for analysis. The existence of a central cavity, internal membrane, crystalline liquid inside and positive pressure at puncture were checked: pulmonary cysts (Fig. 1) in ovine 3 (1), ovine 4 (1), ovine 5 (2), ovine 6 (1) and hepatic cysts ovine 3 (1). None of the cysts contained

Table 1
Location and frequency of cysts found in ovines exposed to eggs of *E. granulosus* naturally aged

Ovine	Total		Liver		Lung	
	No. of cysts	%	No. of cysts	%	No. of cysts	%
3	2	13	1*	6	1*	6
4	5	31	4	25	1*	6
5	7	43	4	25	3**	19
6	2	13	1	6	1**	6
Total	16	100	10	63	6	37

Time of aging for eggs, 41 months; Sarmiento, Province of Chubut, Argentina; $N = 4$.

* Cysts selected for anatomy-pathology study.

** Cysts selected for identification of specific sequences of *E. granulosus*.

protoscoleces. A 33% of the cysts showed cell material of necrotic aspect and refringent corpuscles (pulmonary cysts in ovines 4 and 5).

Histologic characteristics: in the studied samples of control ovine 1, an unspecific chronic peribronchitis was observed. Three cysts developed in the exposed ovines were selected for the confirmatory histologic study; ovine 4: pulmonary cyst, with laminar membrane, with intense lympho-immunoblastic and histiocite pericystic reaction (Fig. 1), and ovine 5: (a) pulmonary cyst, with cystic wall with calcification foci and inflammatory component with multinucleus giant cells, epithelioid and eosinophilous histiocites, (b) hepatic cyst, with central necrosis, non-caseous with eosinophilous and inflammatory reaction of the pericystic area.

Genetic characteristics: for the genetic confirmation study, DNA obtained from pulmonary cysts of ovines 5 and 6 was used. The amplification of a specific fragment of 165 pb through the PCR oEg1 reaction was positive for the DNA isolated from the internal membrane of cyst 5 while a doubtful result was observed in the case of the purified DNA of cyst 6.

The eggs of *E. granulosus* used in this study came from the same lot of mature vermes where fresh eggs were obtained and used to inoculate ten control lambs of the effectiveness confirmation trial of vaccine EG95. The inoculation doses used in both studies were similar, approximately 1200 eggs. When they were fresh, the eggs produced infection in 10/10 of the inoculated ovines, with an average of 24.3 cysts per ovine (Lightowlers et al., 1999). In the present study, it was demonstrated that after 41 months of aging the eggs under environmental conditions of an inferior arid climate infected in 4/4 ovines challenged with 1200 eggs per ovine.

The immune response to infection by taeniids in ovines takes place in two phases: first, *Immunity to the pre-encystment phase*, and second, *post-encystment immunity* (Gemmel et al., 1987). The dead larvae by means of the pre-encystment response are not evidenced in the necropsy, while if they are destroyed by the post-encystment immunity, those larvae appear in the post-mortem evaluation. The fact that developed cysts were found in this study proves that these aged eggs contained an embryo with the capacity to evade the pre-encystment response.

The histologic verification of the presence of laminar membrane in cysts developed from the embryos present in the aged eggs would account for the capacity they had to avoid being destroyed by the pre-encystment immunity and to be able to produce metacestode. The laminar membrane (LM) is involved in the protection of the parasite facing the immune response of the host and prevents the passing of defense cells of the parasite (Thompson, 1995). Holcman et al. (1994) showed that this laminar membrane appears between the 3rd and 5th day of the initial development of the cyst.

From the clinical point of view and from their natural history, hydatid cysts can be considered as either *active cysts* (with crystalline liquid in their inside, fertile or sterile), *cysts in degeneration* (with partial calcification or solidification), or *inactive cysts* (totally calcified or solidified) (Eckert et al., 2001). Taking this classification into account, we could ascertain that the cysts found in the present study belong to the categories of *active sterile cysts*, in the cases of 5/6 found pulmonary cysts and in the hepatic cyst in ovine 3; *cyst in degeneration* in the case of a pulmonary cyst in ovine 5; and *inactive cyst* in the case of a hepatic cyst in ovine 5. None of the cysts found contained live protoscoleces. It should be noted however that no fertile cysts were found and thus the capacity of further infection transmission is limited or nil.

The fertile cysts which have started a degrading process may contain DNA even though the protoscoleces cannot be morphologically individualized as such with the optical microscope due to their advanced state of deterioration. The specific amplification of the *E. granulosus* sequence was observed in the case of pulmonary cyst of ovine 5, while the isolated DNA of pulmonary cyst 6 showed doubtful results. The fact that degraded material was observed with the optical microscope in the liquid of pulmonary cyst from ovine 5 might account for this positive result for *E. granulosus* obtained in the specific identification study. The results of amplification through PCR would be in agreement with the characteristics observed microscopically in the studied cysts.

The laboratory studies give information regarding the survival of eggs under different experimental exposure conditions, and only the field studies include interactions of the diverse meteorological variables

(Sweatman and Williams, 1963). The exposure conditions of our study were in agreement with natural conditions of quasi-continental climate, classified as inferior arid climate (Unesco, 1979; Sánchez Thevenet et al., 2003). From the analysis of the data obtained in similar conditions of study, that is, natural exposure and evaluation of the viability and infectiousness in vivo, we can say that the maximum survival time reported was 120 days for *E. granulosus* in New Zealand (Sweatman and Williams, 1963), and 240 days for *E. multilocularis* in Germany (Veit et al., 1995). The conditions of quasi-continental climate in the Sweatman and Williams (1963) study are comparable to those prevalent in our region.

Gemmel et al. (1987) propose that the processes of maturation and ageing of eggs from taeniids continue in the environment and that the eggs go through different stages during those processes: immature, mature, semi-senescent, senescent, and dead. A mature egg produces a viable larva, evading the immunologic response from the host. An egg in semi-senescent stage overcomes the pre-cystic immunity, settling down in the target organ and starting the development of the larva stage, though later, when this larva is formed, it is destroyed by the post-cystic immunity. It is worth considering that the model of these authors is based on studies on *T. hydatigena*. The characterization of viability of metacestode in *E. granulosus* responds to criteria of morphological integrity and the presence of crystalline liquid inside of them (Lightowlers et al., 1999).

Considering what has been mentioned and assuming the hypothesis of Gemmel et al. (1987) regarding maturation and ageing stages of taeniid is true, the studied eggs of *E. granulosus*, after 41 months of exposure to the natural conditions of the region, would be in the stages of mature eggs and semi-senescent eggs.

The results obtained in the present study show that the embryos contained in the studied eggs which have remained 41 months under natural conditions of inferior arid climate had the capacity to penetrate, settle down and start the post-oncospheral development, processes described by other authors (Heath, 1971; Harris et al., 1989; Thompson, 1995). The embryos of those eggs kept the vital capacities necessary to carry out key steps for the development to metacestode under these natural conditions.

Acknowledgements

To CORFO Regional Sarmiento, who donated the ovines used in this study. To the personnel of Chacra 18, Ganaderia Department, and to Sanitary Technician Carlos Terán, from the ‘‘Programa de Control de la Hidatidosis del Chubut’’, in charge of taking care of the ovines. To Environmental Technician Mario Díaz, Health Department, Province of Neuquén, to Pediatrician MD Pasqual Gregori Roig, MD and Pathologist Trinidad Ojeda Insaurralde Hospital Regional de Comodoro Rivadavia, Chubut, who participated in the bias control instances during necropsy and histologic exam, respectively. To Public Translator Laura Cipolla for the translation of the manuscript.

This study was partially granted by the Programa de Control de la Hidatidosis, Province of Chubut, Argentina, and by the Public Health Chair, Biochemistry Department, Universidad Nacional de la Patagonia San Juan Bosco.

References

Cabrera, P.A., Haran, G., Benavides, U., Valledor, S., Perera, G., Lloyd, S., Gemmel, M.A., Baraibar, M., Morana, J., Maissonave, J., Carballo, M., 1995. Transmisión dynamics of *Echinococcus granulosus*, *Taenia hydatigena* and *Taenia ovis* in sheep in Uruguay. Int. J. Parasitol. 25, 807–813.

Cerrone, G.E., Targovnik, H.M., 2002. Análisis del genoma de *Echinococcus granulosus*. Su aporte al conocimiento y diagnóstico del parásito. In: De Negri, G.M., Elissondo, M.C., Dopchiz, M.C. (Eds.), Situación de la Hidatidosis—Echinococcosis en la República Argentina. Editorial Martín, Mar del Plata, pp. 41–48.

Coman, B.J., 1975. The survival of *Taenia pisiformis* eggs under laboratory conditions and in the field environment. Aust. Vet. J. 51, 560–565.

Coman, B.J., Rickard, M.D., 1977. A comparation of in vitro and in vivo estimates of the viability of *Taenia pisiformis* eggs aged under controlled conditions, and their ability to inmunise againts a challenge infection. Int. J. Parasitol. 7, 15–20.

Eckert, J., Gemmel, M.A., Meslin, F.-X., Pawlowski, Z.S., 2001. WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem Of Global Concern. World Organization for Animal Health (OIE), WHO, Paris.

Gemmel, M.A., Lawson, J.R., Roberts, M.G., 1987. Population dynamics in echinococcosis and cysticercosis: evaluation the biological parameters of *Taenia hydatígena* and comparison with those of *Echinococcus granulosus*. Parasitology 94, 161–180.

Harris, A., Heath, D.D., Lawrence, S.B., Shaw, R.J., 1989. *Echinococcus granulosus* ultraestructure of epithelial changes during

the first 8 days of metacestode development in vitro. Int. J. Parasitol. 19, 621–629.

Heath, D.D., 1971. The migration of oncospheres of *Taenia pisiformis*, *Taenia serialis* and *Echinococcus granulosus* within the intermediate host. Int. J. Parasitol. 1, 145–152.

Holcman, B., Heath, D.D., Shaw, R.J., 1994. Ultraestructure of oncosphere and early stages of metacestode development of *Echinococcus granulosus*. Int. J. Parasitol. 24, 623–635.

Ibrahem, M.M., Craig, P.S., Mc Vie, A., Ersfeld, K., Rogan, M.T., 1996. *Echinococcus granulosus* antigen B and seroreactivity in natural ovine hydatidosis. Res. Vet. Sci. 61, 102–106.

Ilsoe, B., Kyvsgaard, N.C., Nansen, P., Henriksen, S.A., 1990. A study on the survival of *Taenia saginata* eggs on soil in Denmark. Act. Vet. Scand. 31, 153–158.

Kittelberger, R., Reichel, M.P., Jenner, J., Heath, D.D., Lightowlers, M.W., Moro, P., Ibrahem, M.M., Craig, P.S., ÓKeefe, J.S., 2002. Evaluation of three enzyme-linked immunosorbent assays (ELISAs) for the detection of serum antibodies in sheep infected with *Echinococcus granulosus*. Vet. Parasitol. 110, 57–76.

Lightowlers, M.W., Jensen, O., Fernandez, E., Iriarte, J.A., Woollard, D.J., Gauci, G.C., Jenkins, D.J., Heath, D.D., 1999. Vaccination trials in Australia and Argentina confirm the effectiveness of the EG95 hydatid vaccine in sheep. Int. J. Parastol. 29, 531–534.

Maniatis, T., Fritsch, E.F., Sambrook, J., 1989. Molecular Cloning. A laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Sánchez Thevenet, P., Jensen, O., Mellado, L., Torrecillas, C., Raso, S., Flores, M.E., Minvielle, M.C., Basualdo, J.A., 2003. Presence and persistence of intestinal parasites in canine fecal material collected from the environment in the Province of Chubut Argentine Patagonia. Vet. Parasitol. 117, 263–269.

Silverman, P.H., 1956. The longevity of eggs of *Taenia psisformis*. Trans. R. Soc., Trop. Med. Hyg. 50, 7–8.

Sweatman, G.K., Williams, R.J., 1963. Survival of *Echinococcus granulosus* and *Taenia hydatigena* eggs in two extreme climatic regions of New Zealand. Res. Vet. Sci. 4, 199–216.

Thompson, R.C.A., 1995. Biology and sistematics of *Echinococcus*. In: Thompson, R.C.A., Lymbery, A.J. (Eds.), Echinococcus and Hydatid Disease. CAB International, Wallingford, pp. 1–37.

Unesco, 1979. Mapa de Distribución Mundial de las Zonas Áridas. Notas Técnicas No. 7. MAB, París.

Veit, P., Bilger, B., Schad, V., Schäfer, W., Frank, W., Lucius, R., 1995. Influence of environmental factors on the infectivity of *Echinococcus multilocularis* eggs. Parasitology 110, 79–86.

Wachira, T.M., Mac Pherson, C.N.L., Gathuma, J.M., 1991. Release and survival of *Echinococcus* eggs in different environments in Turkana, and their possible impact on the incidence of hydatidosis in man and livestock. J. Helminthol. 65, 55–61.