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Morphological and biological characterization of cell line developed from bovine *Echinococcus granulosus*

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Abstract The taeniid tapeworm *Echinococcus granulosus* is the causative agent of echinococcal disease, a major zoonosis with worldwide distribution. Several efforts to establish an in vitro model of *E. granulosus* have been undertaken; however, many of them have been designed for *Echinococcus multilocularis*. In the present study, we have described and characterized a stable cell line obtained from *E. granulosus* bovine protoscoleces maintained 3 yr in vitro. Growth characterization, morphology by light, fluorescent and electronic microscopy, and karyotyping were carried out. Cell culture origin was confirmed by immunofluorescent detection of *AgB4* antigen and by PCR for the mitochondrial cytochrome c-oxidase subunit 1 (DCO1) gene. Cells seeded in agarose biphasic culture resembled a cystic structure, similar to the one formed in secondary hosts. This cell line could be a useful tool to research equinococcal behavior, allowing additional physiological and pharmacological studies, such as the effect of growth factors, nutrients, and antiparasitic drugs on cell viability and growth and on cyst formation.

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Introduction

The taeniid tapeworm *E. granulosus* is the causative agent of echinococcal disease, a major zoonosis with worldwide distribution (D'Alessandro 2002; Cabrera et al. 2003; Craig et al. 2007).

Serum reactivity to *E. granulosus* antigens is prevalent in human populations in Latin America, reaching 2% in Southern Argentina and 5.8% in the south of Brazil. A considerable body of information on the biochemical, molecular, and immunological features of echinococcosis has been gathered over the past two decades. However, recording experimental molecular data on physiology, genetics, and molecular biochemistry has been difficult without an established cellular laboratory model.

Many in vitro models have been developed for *E. multilocularis*, such as isolated germinal cells (Dieckman and Frank 1988; Furuya 1991; Yamashita et al. 1997), metacestode cultures (Hemphill and Gottstein 1995; Hemphill et al. 2002; Spiliotis et al. 2004), and primary cell cultures from metacestodes line (Spiliotis and Brehm 2008). Cell cultures and other in vitro models for *E. granulosus* are scanty. Fiori et al. (1988) cultured cells from hydatid cyst. Smyth et al. (1967) and Casado and Rodriguez Caabeiro (1989) cultured protoscoleces (*pe.*). Heath and Lawrence (1976) cultured oncospheres and obtained young cysts, and Liu et al. (1998) established a cell line from *E. granulosus* cyst germinal layer grown in sheep liver. These models have mainly served to study larval development, pharmacological drug effects, and in vitro parasite–host cell interactions. Recent in vitro models

have also been developed for molecular genetic studies and genetic manipulation.

An interesting aspect is the ability of *Echinococcus* to renew structures and to form mature cells from stem cells by cellular differentiation pathways. Galindo et al. (2003) studied the germinal layer of *E. granulosus* to elucidate how they develop mature *pe.* from buds. They also demonstrated active DNA synthesis in mature *pe.* bodies with suckers. The reverse process—to obtain pluripotent cells from complex structures or mature cells by cellular de-differentiation—has been accomplished so far in *E. multilocularis* by Spiliotis and Brehm (2008).

In the present study, we have established a cellular model of *E. granulosus* from bovine *pe.* that can be indefinitely cultured in an axenic controlled medium, develop cystic structures, and respond to stimuli in the absence of host cells, thus providing the opportunity to characterize genetic plasticity and physiology. Cells were obtained from bovine *pe.* by enzymatic digestion. They were then cultured for 3 yr, frozen, and later thawed. After continuous culture for 18 mo, cells were seeded under defined biphasic conditions, obtaining cystic-like structures. Cells were then characterized by routine histology techniques, electronic microscopy, and PCR. Further characterization was performed by immunofluorescent detection of *B4* antigen (*AgB4*) expression. Chromosome morphology and distribution were also studied. This cell line was named EGPE.

Materials and Methods

Primary cell culture. E. granulosus protoscoleces from bovine liver (courtesy of Dr. Guillermo Denegri, Universidad Nacional de Mar del Plata, Argentina) were rinsed with Tyrode solution containing 300 μg penicillin/500 μg streptomycin and 1.4% sucrose. Enzymatic digestion was performed using 2.5 U/ml papaine (Sigma, St. Louis, MO, USA) while stirring at 25°C for 1 h. Samples were centrifuged at 1,500 rpm and the pellet was diluted in medium 199 (Sigma), 1 mM sodium pyruvate (sodium salt extra pure, Anhedra, Beijing, China) and 78 μg/ml β-mercaptoethanol (Merck, Darmstadt, Germany) at pH 7.9. Cells were then incubated in 75-mm diameter Petri dishes in a bacteriological incubator without CO_2 at 35°C (San Jor, Buenos Aires, Argentina). Half the medium was replaced weekly and cell viability was tested with eosin. Cells were later diluted monthly in 1:3 and grown with and without the addition of 1–10% biotechnological fetal bovine serum (FBS, Internegocios SA, Buenos Aires, Argentina). Cell culture was continuously maintained and aliquots were frozen in liquid nitrogen with 10% dimethylsulfoxide (Sigma) and 20% FBS-supplemented medium. Cell growth was studied under different culture conditions, with different concentrations of FBS and also without FBS and incubated at 37°C in 5% CO_2/95% air (Sanyo, MCO 17AC, Gunma-Kan, Japan). Cell culture contamination screening for bacteria was performed after cell centrifugation in different bacterial growth media and by Gram staining. Absence of cell contamination has also been confirmed by DNA examination during karyotype studies and by acridine orange observations.

Cell viability and cell growth. Viability was studied by dye exclusion viability test routinely performed with 1% water-soluble eosin. Test was performed three times with acridine orange fluorescent dye in cells grown with and without 10% FBS for 3–4 d. They were centrifuged at 1,500 rpm and pellets were diluted in 1% Acridine orange in PBS. Acridine orange (Sigma) was incubated for 5 min. Mitochondrial activity was studied with 1/100 MitoRed (Molecular Probes, Eugene, OR, USA) in PBS according to supplier's guidance. Afterwards, cells were observed under fluorescence microscope. Cell growth was studied by seeding 10^3 cells in each well of a 24-well cell culture plate (Falcon, Oxnard, CA, USA). Every 2 d, cells from three wells were counted using the dye exclusion test.

Histological studies. Agarose cell colonies and suspended cells were fixed with 4% neutral formaldehyde, dehydrated, and embedded in paraffin. Paraffin was then cut at 8–10 μm and stained with hematoxylin–eosin or periodic acid solution (PAS) in order to study membrane disposition.

Electron microscopy. Cells were collected after 72 h of growth and centrifuged at 4,000 rpm for 2 min. The pellet was fixed with 2.5% glutaraldehyde (Merck) in buffer phosphate at pH 7.4 for 2 h at 4°C. Afterwards, the pellet was washed with PBS and post-fixed with 2% OsO_4 at 4°C for 90 min. Counterstaining was performed with 2% uranile acetate. Other samples were processed to study acellular membrane disposition and composition. Collected cells without centrifugation were fixed with 2.5% glutaraldehyde/8% tannic acid (Merck) and post-fixed with 1% OsO_4 or with 0.15% red ruthenium (Sigma) instead of tannic acid. Samples were included in DER 732 resin, cut into ultrathin sections, and stained with methachromatic blue tolouidine and blue methylene or used for transition electronic microscopy with Jeol electronic microscopy (Tokyo, Japan) in Microscopy Electronic laboratory of INTA Castelar, Buenos Aires, Argentina.

Immunofluorescence for AgB4 antigen detection. Cells grown in culture for 3 d were seeded in an Eppendorf tube, rinsed with PBS, and centrifuged at 1,500 rpm for 5 min. Cells were incubated in PBS and 1/800,000 dilution of a polyclonal rabbit antibody against a subunit of recombinant *AgB4* (a gift from Dr Mara Rosenzvit, Universidad de

Buenos Aires, Argentina). We used a sample incubated with 0.1% non-immune rabbit serum as a technical control. Anti-rabbit IgG FITC (Sigma) diluted 1/100 was used to label samples and controls. Incubations were performed for 0.5 h each. Cells were then washed twice with PBS and counterstained with 0.1% propidium iodide (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were spread onto a slide mounted with 10% glycerol–PBS and observed with Arcano® microscope (Beijing, China) supplied with red and green fluorescence filters. Pictures were taken with a digital camera with zoom.

PCR for mitochondrial DCO1. PCR was performed twice: 2.5 and 10 mo after primary culture. We detected the expected 285-bp specific band that corresponds to a fragment of the mitochondrial cytochrome *c*-oxidase subunit 1 gene DCO1 (5′-TCATATTTGTTTGAGTATTAGTGCTAATTTTGATGCGTTTGGGTTCTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGTAGCAGGGTTTGGGGTCATCATATGTTTACTGTTGGGTTGGATGTGAAGACGGCTGTTTTTTTTAGCTCTGTTACTATGATTATAGGGGTTCCTACTGGTATAAAGGTGTTTACTTGGTTATATATGTTGTTGAATTCGAGTGTTAATGTTAGTGATCCGGTTTTGTGATGGGTTGTTTCTTTTATAGTGTTGTTTAC-3′).

It was contained in a final volume of 25 µl of a buffer reaction containing 0.2 mM dNTP, 2 mM $MgSO_4$, 1 µM primers DCO1F (5′-TCATATTTGTTTGAGKATYAGTKC-3′), and DCO1R (5′-GTAAATAAMACTATAAAAGAAAYMAC-3′) in the presence of 0.5 U DNA polymerase (Taq Platinun®, Invitrogen, Carlsbad, CA, 5 U/µl). Positive controls were performed with fresh *pe*. washed twice with PBS. Negative controls were performed with porcine liver DNA. Washed *pe*. and cells were boiled 10 min in water and then DNA was seeded for PCR reaction, with the first step performed at 94°C for 2 min, followed by 40 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 68°C, and 10 min at 68°C as extension time (Cabrera et al. 2002).

Karyotype and modal chromosome distribution studies. Cells were cultured for 48 h. Afterwards, 0.2 mg/ml colchicine (Carlo Erba, Milan, Italy) was added and cells were incubated for another 4 h. Cells were later centrifuged at 1,000 rpm for 5 min at room temperature. Then, they were resuspended three times in 75 mM KCl for 15 min and later fixed with methanol/acetic acid (3:1). Fixed cells were spread onto slides, stained with 0.4% Giemsa (Fernandes et al. 2008), and observed with 1,000-fold magnification (Arcano stereomicroscope, Beijing, China 211). Pictures were captured, processed, and analyzed with ADOBE Photoshop CS2 software. Forty metaphases were counted and evaluated.

Tridimensional model or cystic-like structures from continuous cell culture. Agarose (Merck) was prepared at 2% in 5 mM HEPES/5 mM fosfate buffer, pH 7.4, 30 µg/100 ml hemine (Sigma) and 0.5 ppm $ZnCl_2$. Liquid phase was performed in culture medium. Cells were then seeded onto agarose (solid phase) or suspended in agarose at 37°C before gelation. Then, liquid medium was added. Structure formation was evaluated for 1 wk.

Results

Primary cell culture, growth, viability, and morphology. Digestion of *pe.* with papain gave rise to cell clumps which were kept in serum-free medium for 3 mo in order to restrict the growth of any mammalian cell contamination. Cells grew slowly in serum-free medium and were diluted once a month. They were later adapted to culture medium. As a result, we found that the addition of 10% inactivated FBS yielded bigger cells than in the culture medium alone. Figure 1 shows totally adapted in vitro cell growth after

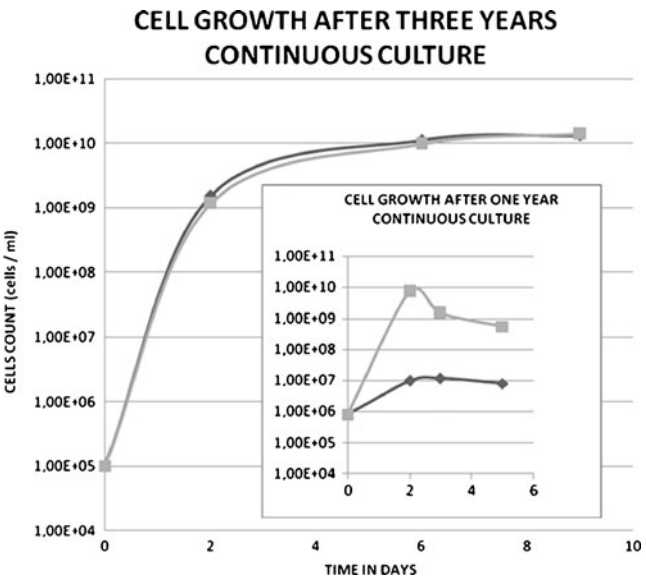

Figure 1. Cell culture conditions effect on growth. Growth adapted in vitro cells after 3 yr of continuous culture. Every *point* represents mean values of three wells in two independent experiments. Cells are incubated in medium 199 with 10% FBS, with (*gray line*) or without (*dark line*) 5% CO_2 incubator. Adapted cells had a fast logarithmic growth for 2 d and then a second logarithmic phase up to day 4. Afterwards, cells decreased growth rate until they reached a plateau. *Insert* shows cell growth after only 1 yr of in vitro continuous culture, while cells were not yet fully adapted. Cells incubated with (*gray line*) and without (*dark line*) addition of 10% FBS. Regardless of differences in proliferation rates among both groups, cells picked up after 2 d of incubation ($p<0.001$), but lacked a second logarithmic growth phase, which appeared only after 3 yr of culture. Total cell adaptation was reached even later.

3 yr of continuous culture in medium 199 with 10% FBS with and without 5% CO_2/95% air incubator. Adapted cells had a fast logarithmic growth for 2 d and then a second logarithmic phase up to day 4; afterwards, cells reached a plateau. Insert shows in vitro cells' growth after only 1 yr of continuous culture, while cells were not yet fully adapted, cultured with and without the addition of FBS. Regardless of the differences in proliferation rates among both groups, cells picked up after 2 d of incubation ($p < 0.001$), but lacked a second logarithmic growth phase, which appeared only after 3 yr of culture. Total cell adaptation was reached even later.

The most remarkable characteristic was that these cells never attached to the flask surface and grew better in Petri dishes and in multiwell plates than in tissue culture flasks. They formed a suspended monolayer that attached to an acellular layer formed after a week of culture, as observed in Fig. 2. Figure 2*A* shows cells in primary culture while Fig. 2*C* shows cell aggregates after 3 yr of continuous culturing. Figure 2*B*, *D* shows H&E-stained cells obtained from primary cell culture (panel *B*) and after 3 yrs of continuous culture (panel *D*).

Morphology studies were performed with vital dyes after 3 yr of continuous culture: acridine orange for visualizing nucleus and cytoplasm and MitoRed for functional mitochondria. Figure 3*A* shows mitochondria distribution and Fig. 3*B* shows nucleus/DNA and cytoplasm/RNA. Both micrographs show grouped cells that seem to form a syncytium. Mitochondria are relatively large compared to cell size.

Results shown in Fig. 4 from electron microscopy of cells in mitosis during early metaphase (Fig. 4*B*) and cytokinesis (Fig. 4*A*) suggest high biosynthetic cell activity and secretion (Fig. 4*C*, *D*). Cells appear surrounded by a membrane, forming cell aggregates. Indeed, acridine orange stain suggests a syncytium organization. Ultrathin microscopy of cell monolayer without disruption by centrifugation and

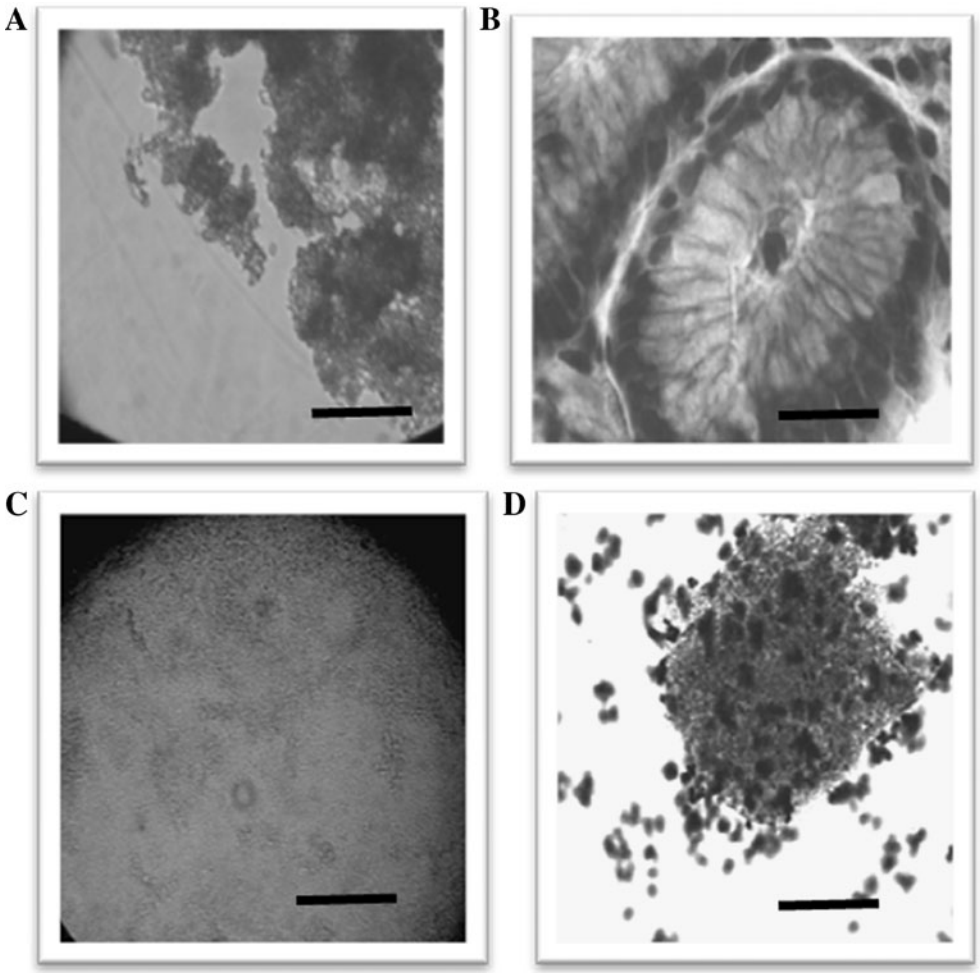

Figure 2. Cells form aggregates in vitro. Morphology of EGPE cell monolayer in the plate culture. (*A*) Primary culture: cells grow in aggregates without attaching to plate surface, at 100-fold magnification. (*B*) Monolayer of A stains with H-E, characterizing cell aggregates (with zoom, ×1.5 zoom). (*C*) Cell monolayer appearance after 3 yr of culture. *d*, *h*, *e* Stains characterizing cell aggregates at the same time of as in (*C*). Cell aggregates changed their appearance according to time spent in culture. *Line* represents 100 μm for images (*A*) and (*C*) and 25 μm for image (*B*) and 10 μm for image (*D*).

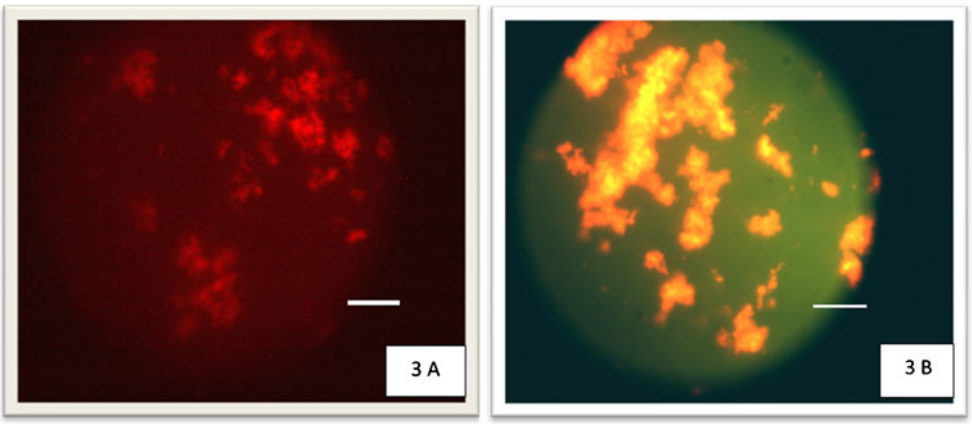

Figure 3. Fluorescent dye confirms cellular aggregates. Vital dyes stain of mitochondria, nucleus, and cytoplasm from grouped EGPE cells after 3 yr of continuous culture. *A* Mitochondria stained with Mitored (*bright red*) after 30 min of incubation at 37°C. *B* Nucleus (*light green*) and cytoplasm (*orange*). Cells are grouped and seem to form a syncytium. Mitochondria are relatively big compared to cells size. *Line* represents 50 μm.

stained with ruthenium red shows a high glycoconjugate acellular membrane surrounding the cells (Fig. 4*E*) and high osmium-affinity bodies (Fig. 4*F*). The fact that these samples showed figures compatible with dead cells (data not shown) suggests that this membrane could be formed—at least in part—by these dead cells and by cellular secretion, as has been seen in mammalian epithelia.

The confirmation that EGPE cell line actually came from *E. granulosus* was demonstrated studying *AgB4* antigen expression by immunofluorescence and DCO1 mitochondrial sequence by PCR amplification. Figure 5*A* shows antigen expression while Fig. 5*B* shows a negative image for *AgB4* and positive nucleus counterstain in the same slide, which confirms antigen expression in cellular structures. PCR also confirmed *E. granulosus* cell origin by detection of the fragment of the mitochondrial cytochrome *c*-oxidase subunit 1 (DCO1) gen found in fresh *E. granulosus pe*. and in EGPE cells (Fig. 5*C*).

Karyotype and modal chromosome distribution studies. Cell line was also characterized by metaphase analysis and modal chromosome distribution, both determined in cells in continuous culture after 2 yr. Metaphase chromosomes scored between 190 and 265, with a modal value around 250 per metaphase in 65% of the cases (Fig. 6*A*). Besides the high DNA content, another outstanding finding was the presence of terminus–terminal chromosome unions in 90% of chromosomes *in tandem* (Fig. 6*B*). End and/or end associations formed tandems of three to six chromosomes. We also found that these associations were not the results of artifacts since tandems were present in all methaphases regardless of the culture or harvest parameters (culture time from 48 to 72 h, colchicine incubations from 1 to 4 h, or the renewal of all solutions used in culture or harvesting). Chromosome morphology distribution according to centromere location was 70% metacentric, 10–15% acrocentric, and 15–20% submetacentric (Fig. 6*B*).

Cell growth in agarose showed cystic organization. After 2 yr of continuous cell growth, cells were seeded in biphasic media to assay if they would reorganize into structures similar to those observed in secondary hosts. Agarose was selected among other polymers due to its neutral properties and its low interaction with biological molecules. Agarose can additionally be enriched with different substances, which allowed us to study the effect of salts, growth factors, lectins, and other molecules on structure formation. Tests took place varying agarose concentrations and supplemental factor concentrations (Fuchs 2008). Just as vegetable seeds do in soil, our cells seeded on agarose, forming a cystic-like colony. This structure was maintained for up to 1 wk, although many cells failed to integrate into this structure and remained in the culture's liquid phase. Figure 7*A*, *B* shows structure 24 h and 8 d after seeding, respectively. When cells were seeded into the agarose matrix—instead of on it—not only more cellular colonies were obtained but also more cells were integrated into the cyst-like structure (Fig. 7*C*, *D*). Colony sizes varied and were easily observed at 100-fold magnification at different matrix agarose levels. Figure 7*E*, *F* shows light microscopy images of cell organization inside the cysts, surrounded by a PAS-positive membrane. Videomicroscopy and Fig. 8, show how cells move inside the membrane, just as they did in liquid culture, which demonstrates liquid accumulation inside the cystic structure.

Discussion

In the present study, we have described a monolayer cell line from *E. granulosus* bovine protoscoleces (EGPE) and found that when seeded in biphasic culture, these cells develop a cystic structure, similar to that of metacestodes in secondary hosts.

There have been several efforts to establish an in vitro model of *E. granulosus* since physiological, biochemical,

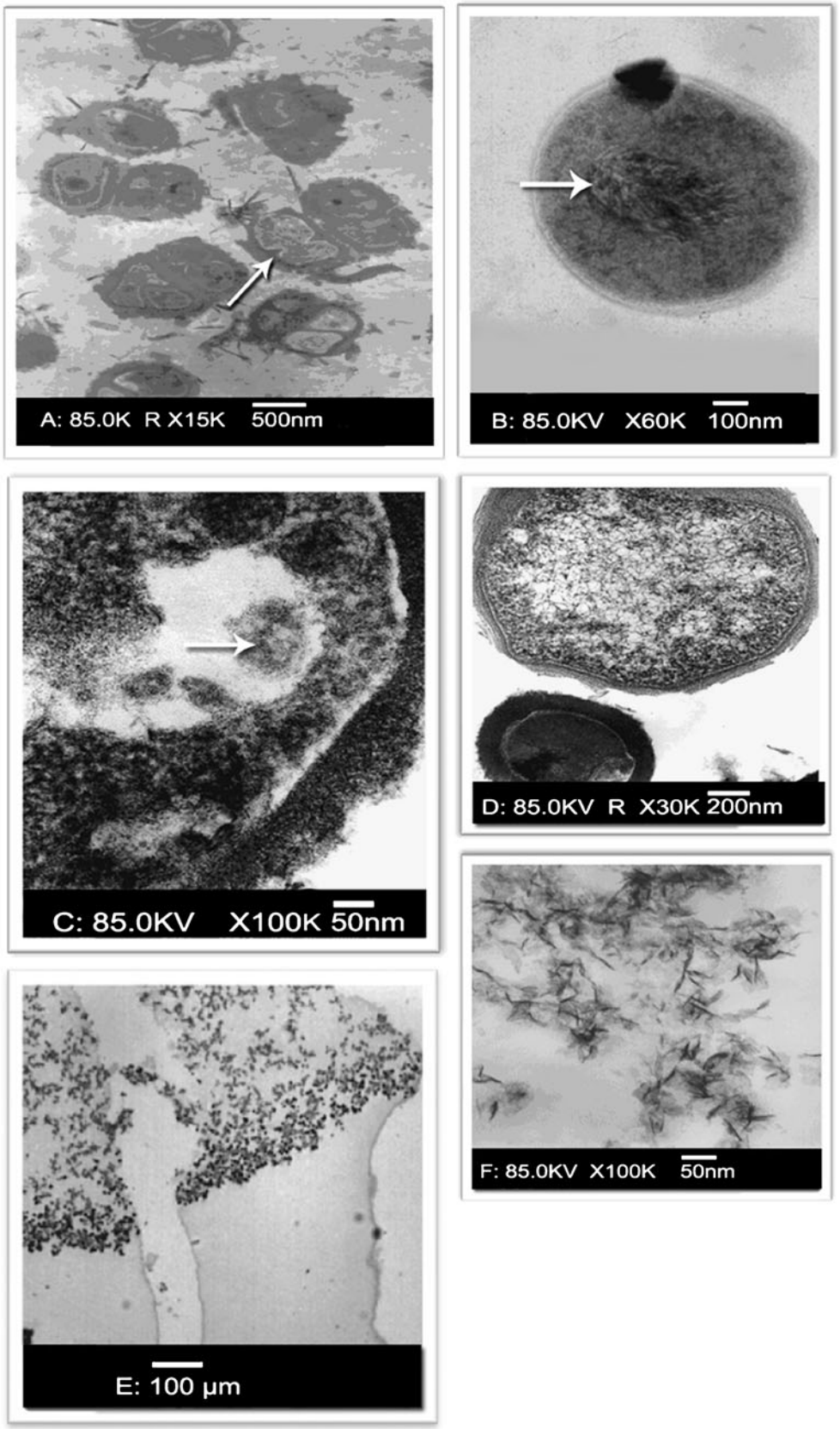

Figure 4. Electronic microscopy (E.M.) shows details of the established EGPE cell line. (*A*) Group of differently shaped cells surrounded by a membrane. *Arrow* shows a cell in cytokinesis. (*B*) Dividing cell in early metaphase, at the beginning of chromatid migration. (*C*) Biosynthetic cell activity. *Arrow* shows electrodense material in cell cytoplasm, approaching cell membrane. (*D*) Cell shows details of cytoskeleton structure and electrodense granule accumulation in cell periphery. (*E*) Stereoscopic microscopy (100-fold magnification) of ultrathin section processed for E.M. and stained with ruthenium red, showing a border between the cells and the rutheniun red-positive membrane. (*F*) E.M. of membrane stained with ruthenium red and osmium, presenting electrodense material accumulated with heterogeneous distribution.

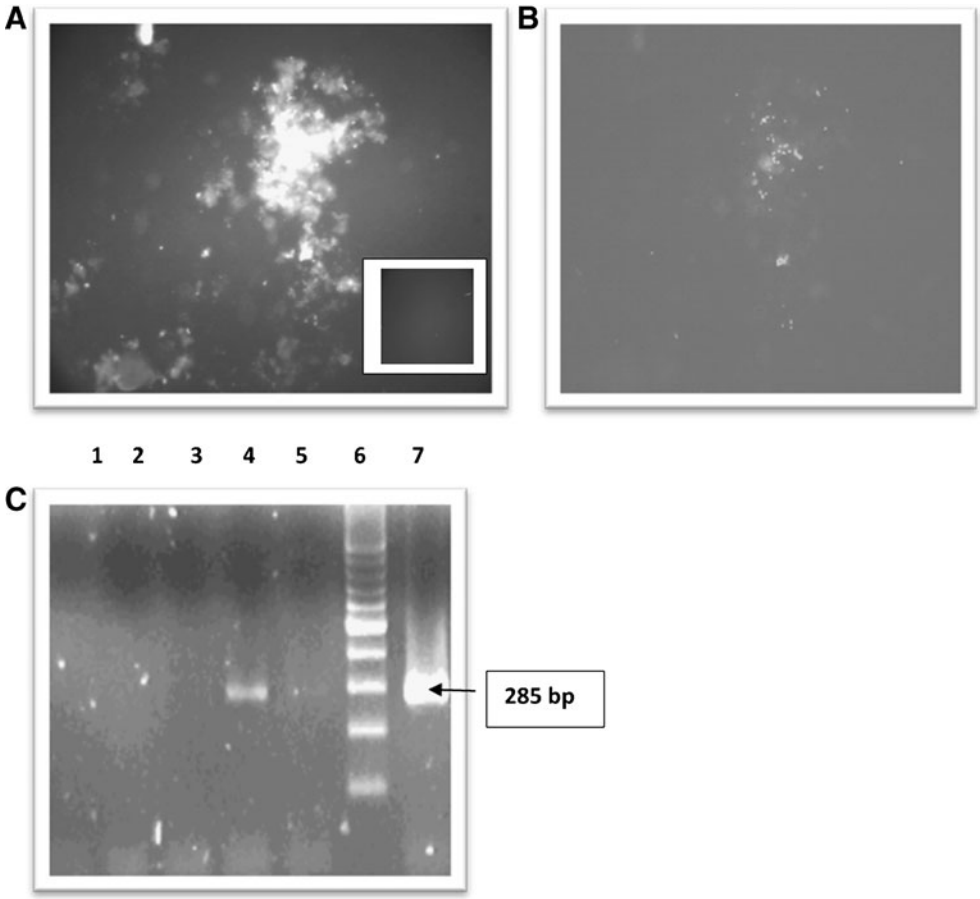

Figure 5. Immunological and molecular identification of cell origin. *A*, *B*: Samples labeled with polyclonal rabbit antibodies against recombinant *AgB4* and anti-rabbit IgG FITC and counterstained with propidium iodide. *A*: *AgB4* expression in regions of grouped cells. *Insert* in (*A*) shows negative methodological control. *B*: Counterstain shows propidium iodide distribution (nucleus). *C*: PCR amplification of fragment of the mitochondrial cytochrome *c* oxidase subunit 1 (DCO1) gen in EGPE cell line after 10 mo of incubation. *Lanes 1* and *2* are negative controls; *3* DNA from porcine liver (30 ng); *4* DNA from EGPE cell line (44 ng); *5* running buffer; *6* a molecular weight marker of 100-bp DNA ladder; *7* fresh bovine *pe.* DNA (50 ng, positive control).

and molecular studies require system isolation. Many were short-term primary *E. granulosus* cultures at different evolution stages: *oncosphere* activation and development (Heath and Lawerence 1981); monolayer culture from metacestode germinal membrane (Liu et al. 1998) which produced cells attached to a plastic plate (Fiori et al. 1988); and monophasic culture of *pe*. (Ponce Gordo and Cuesta Bandera 1995) which showed strobilar differentiation of *pe.* cultures when seeded in disphasic medium, with a solid phase resulting from serum coagulation (Smyth 1990).

Cell isolates were obtained from *E. granulosus* protoscoleces using heavy treatment with papain since

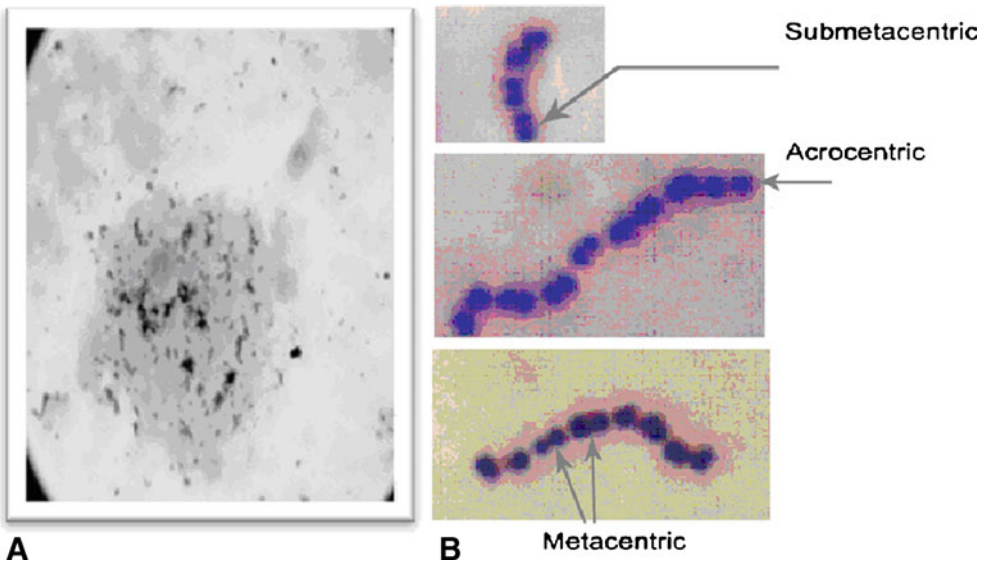

Figure 6. Karyotype and chromosome morphology. (*A*) Nucleus in methaphase with high DNA content, at 400-fold magnification, plus zoom. (*B*) Chromosome morphology, with terminus–terminal unions between chromosomes (1,000-fold magnification, plus zoom).

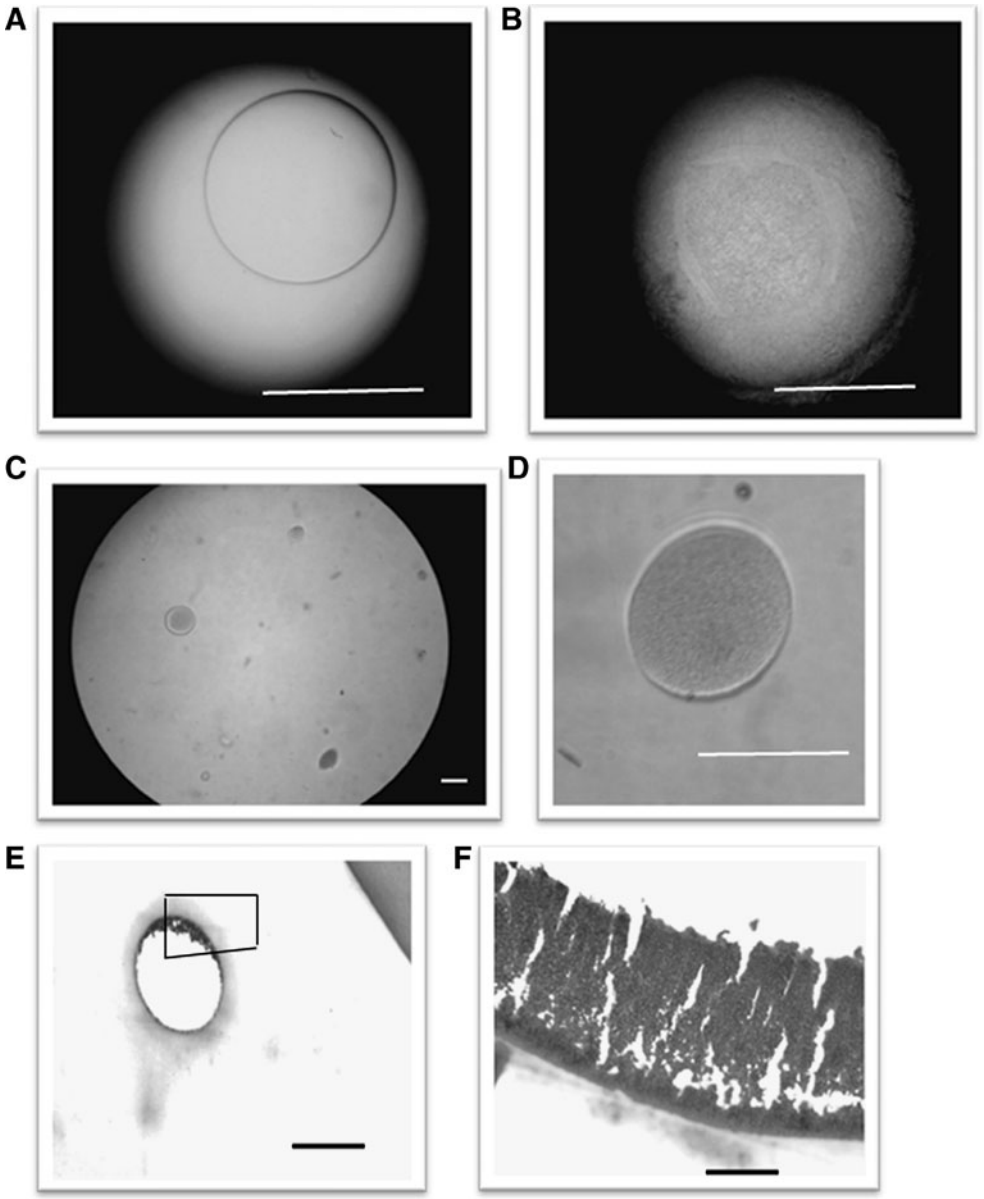

Figure 7. Cystic-like structure formed by EGPE cells cultured in biphasic medium with 2% agarose and culture medium with 10% FBS. *A*, *B*: Structure formed when by EGPE cells seeded on agarose, at 100-fold magnification. Large cystic structure formed after 24 h of incubation. *B*, Same structure after 8 d of incubation, showing an opaque cellular formation surrounded by a bright acellular membrane. *C*, *D*, Colony structures when formed by cells seeded into agarose. *C*, Cystic-like colonies, seen with 100-fold magnification. *D*, Same, seen with 400-fold magnification. *E*, Transverse section of cystic-like structure surrounded by membrane, seen with 100-fold magnification. *f* Detail labeled in (*e*), seen with shift-periodic technique (*PAS*), with 1,000-fold magnification, plus zoom. *A*, *B* *Line* represents 200 μm. *c*, **d** *Line* is 50 μm. *e Line* is 200 μm. *f Line* is 20 μm.

trypsin treatment was ineffective (personal communication). During the 3 yr of continuous culture, cell growth conditions regarding temperature and medium requirements were very strict. Cells were very sensitive to temperature changes, with arrested growth and death at temperatures below 35°C (data not shown). Medium requirements were specific for β-mercaptoethanol and sodium pyruvate which protect cells from free oxygen radicals formed during respiration and cell metabolism. Additionally, sodium pyruvate stimulates aerobic metabolism and ATP production under aerobic and anaerobic conditions. Medium 199 was chosen for its use in normal undifferentiated mammalian cell culture, maintaining undifferentiated state. Cells were grown under two sequential conditions: first in a dry bacteriological incubator for 2 yr and later on in a 5% CO_2/95% air incubator. Changes in atmosphere conditions did not modify growth rates, as has been shown in Fig. 1. Contrary to *E. multilocularis*' in vitro growth, *E. granulosus* was able to grow in the presence of oxygen and not only under strict reducing conditions. *E. granulosus*' growth in the presence of 10–20% O_2 was described by Smyth (1967). Moreover, this value corresponded to the 21% found in atmospheric O_2 concentration. The addition of FBS improved cell growth regardless of its concentration (1–10%) and has been hypothesized to have a nutrient-trophy effect, for example as a source of hexosamines (Heath and Osborn 1976). Indeed, FBS contribution to growth factors has been described for the EGF receptor family in *E. multilocularis* (Brehm and Spiliotis 2008; Brehm et al. 2006) and also for sequences of BMP 2/4, described in *E. multilocularis* and in *E. granulosus* (Fernández et al. 2002). Serum factors additionally seem to be necessary for consistent membrane formation, as previ-

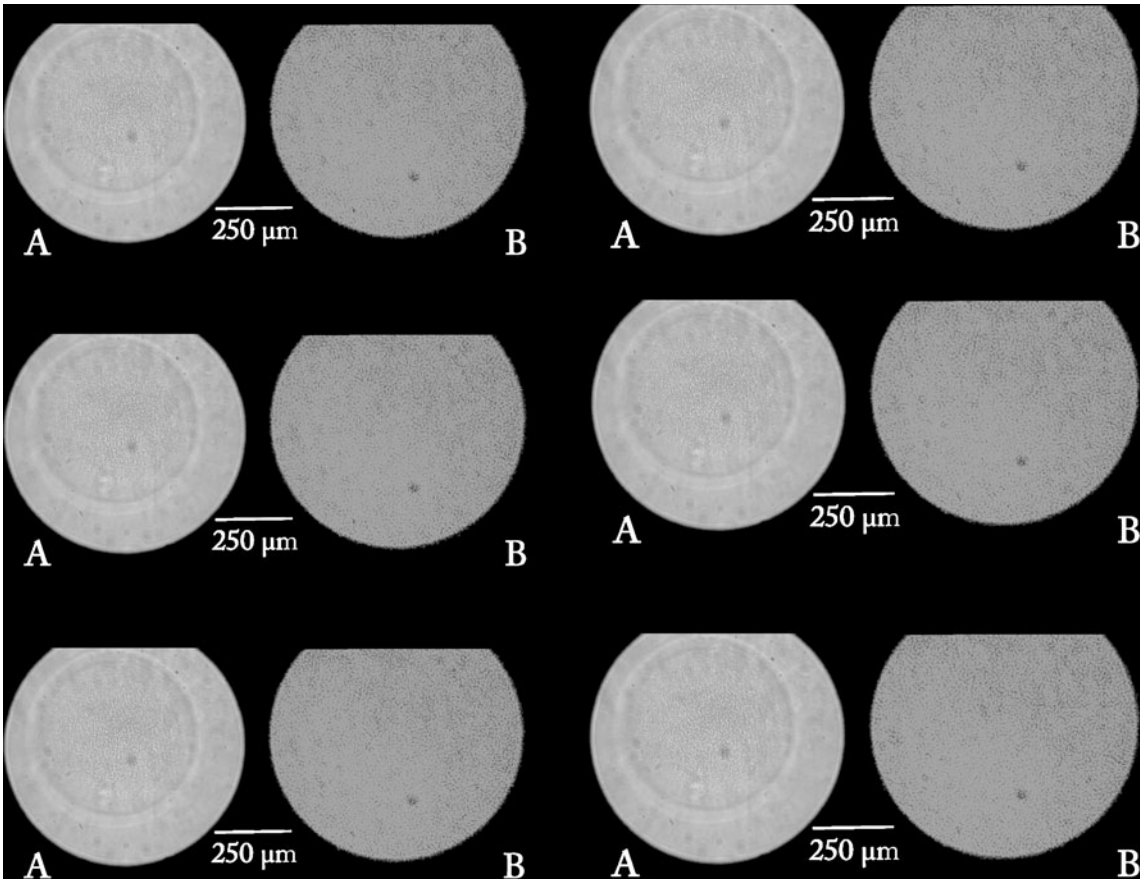

Figure 8. EGPE colonies are cystic. Videomicroscopy images from inverted microscope of cell culture, taken at every second. *A* Cells tethering inside liquid collection with a cystic-like structure in a semisolid support of agarose. *B* Cells tethering inside culture medium (without agarose). Sequence begins at the *upper left corner*, runs down to the *bottom*, and then continues on the *upper right corner*.

ously hypothesized (Smyth 1990). Nevertheless, EGPE cell line can be cultured without serum, indicating that autocrine growth factors released from cells are enough to maintain them alive, although with a slower growth rate, reaching stationary phase at the same time as cells cultured with FBS, but with a lower cell number.

Parasite structure consists of three layers: an inner germinal layer that is surrounded by a tough elastic non-cellular laminated layer, which in turn is surrounded by a host-produced adventitial layer. Protoscoleces are clones formed by a budding process generated in the germinal layer. Studies about their development have shown a non-homogeneous cell distribution into buds accumulated in peripheral areas. There is also information on how in nascent *pe.* nuclei are concentrated in suckers and in rostelar pads and hooks, while in mature *pe.*, nuclei are found adjacent to the tegument, and no cell proliferation is observed after grown buds elongate (Martínez et al. 2005). Moreover, greater proliferative activity was described in *pe.* than in germinal layer, being found mainly in the body and less in neck, rostellum, and suckers; 7% free *pe.* presented DNA synthesis (Galindo et al. 2003).

In this model, we observed a heterogeneous cell distribution over an acellular membrane. The cellular layer looks like a plane *pe*. or bud or like a disorganized germinal laminae membrane structure. Long-term EGPE cell culture, over a month, developed heterogeneous cell aggregates from many sectors of the supporting membrane, seeming to form a syncytial structure.

Ultrastructure studies performed on EGPE cells showed grouped cells with prominent nuclei and cytoplasm containing electronegative and electrodense material, perhaps in the process of secretion. Nevertheless, no defined organelles were observed, just as in *oncosphere* epithelium (Harris et al. 1989). Cytoskeleton ultrastructure is more compatible with epithelial cells rather than with muscular cells. For all these reasons, we believe that these cells may correspond to cells located near the tegument, a site of DNA synthesis that is associated to the in vitro larval development of protoscoleces (Galindo et al. 2008). Structure and organization of germinal membrane is similar to that of adult cestode tegument (Bortoletti and Ferretti 1978). This same structure and organization may be found not only in *pe.* tegument but also in broad capsule walls,

with the difference that tegument polarity appears to be inverted since microtriches are on the inside. The exocytosis of granules suggested in ultrastructure studies could be ascribed to that performed by these cells into the laminated layer adjacent to cysts in culture (Richards et al. 1983). The acellular membrane formed in culture may correspond to the in vivo laminar basal membrane due to its polysaccharide composition, demonstrated by ruthenium red and PAS stain. As explained before, serum factor was necessary for its adequate formation, as previously hypothesized (Martínez et al. 2005).

In order to characterize growing cells, an indirect immune detection for antigen B using polyclonal rabbit antibody against recombinant *AgB4* was performed. Antigens B and 5 detection is the overall accepted basis for human hydatidosis immunodiagnosis. Antigen B is a strongly immunogenic, polymeric, thermostable lipoprotein. Its role in parasite biology has not yet been completely elucidated. Antigen B expression is associated with immature stages in *E. multilocularis* (Carmena et al. 2006; Mamuti et al. 2006). Its main function seems to be the inhibition of elastase activity, though it seems to have no effect on trypsin or chemotrypsine activity (Shepherd et al. 1991). EGPE cells express strong antigen signals seen mainly in groups of cell membranes formed in vitro. This could indicate not only primary cells' origin but also immature state. There is certain discrepancy among authors regarding antigen B localization. It has been described in germinal membrane, in brood cyst wall capsules, and in non-degenerated *pe.* parenchyma and pedicle (Sanchez et al. 1991). Further studies localized it in PAS-positive membranes (Yarzabal et al. 1977). Accordingly, ultrastructural studies showed this antigen to be found in interstitial localizations, connective regions, and disorganized areas in *pe.* tegument surface (Sanchez et al. 1993). Finally, the molecular characterization detected the fragment of the mitochondrial cytochrome *c*-oxidase subunit 1 (DCO1) gen, indicating conclusively the parasite origin of the EGPE cell line (Cabrera et al. 2002).

Cells were also characterized by karyotype and chromosome disposition. It is difficult to determine whether these cells are hyperhaploid or hyperdiploid, considering such a high chromosome number (190–265). Up to 400 chromosomes can be found in tumoral cells (Li 1996) due to cell endoreduplication. This same mechanism is also found in normal platelet development, triggering cell differentiation (Lordier et al. 2008). In *Caenorhabditis elegans*, for example, this mechanism seems to be involved in worm maturation (Hedgecock and White 1985). *E. granulosus* protoscoleces maturation, however, is not associated with an increased chromosome number or DNA content (Bortoletti and Ferretti 1978). Furthermore, considering the large number of cells growing very close together, forming a syncytium, it is possible that cells could be cycling. This multinucleate mass of cytoplasm produced by cell merging could result in a multinuclear image, showing a large number of chromosomes.

Few studies have been published on *E. granulosus* karyotype. Authors have reported a cell line derived from the germinal layer, with a chromosome number that varies from 40 to 90, with a median of 42–44, studied in a 2-yr cell line showing acrocentric and submetacentric morphology (Fiori et al. 1988). In cytogenetic studies performed on germinal membrane *E. multilocularis* cell culture, after 40 time passages, the established cell line exhibited 91–100 chromosomes classified into three main types: telocentric, subtelocentric, and metacentric (Furuya 1991). However, other publications described 24 chromosomes (Raucsh and Rausch 1981). In *Cyathocephalus truncates*, diploid karyotypes with 18 chromosomes, mainly acrocentric and submetacentric, have been reported, though some cells also present polyploidea (Petkeviciüte 1996). In *Eubothrium salvelini*, *Eubothrium crassum*, and *Eubothrium* sp., eight pairs of chromosomes, two of them metacentrics, have also been found (Petkeviciūte and Bondarenko 2001). In *C. elegans*, a nematode studied as a laboratory model, chromosome telomeric fusion has been described, just as in EGPE. Telomeric fusion could be ascribed to telomere erosion due to a variety of mutations that result in telomeric deficiency and genomic instability (Hodgkin 2005). The geometry of replication machinery suggests that telomeric association has to be resolved in order to guarantee progression throughout the cell cycle (Dynek and Smith 2004). However, in this particular cell model, all metaphases showed associations and there was no delay in cell cycle. There are two possibilities open for exploration in future studies: one is that chromosomes could replicate individually and then collide to form end-to-end association after S phase; another possibility, described in *C. Elegans*, is that chromosomes could be polycentric or holocentric—there are many points to be attached to spindle—and after many passages, there is telomeric erosion resulting in a great number of fusions that stably propagate.

Cells formed cystic-like structures in 2–3% agarose. Agarose was chosen because of its advantages over other solid phases, such as feeder layers, serum coagulation, or agar. Additionally, because of its neutral nature, agarose can be supplemented under controlled conditions with factors, salts, molecules, and drugs, allowing cell biology studies. Our study shows that EGPE cells do not need feeder layers to develop cyst-like structures, contrary to *E. multilocularis* isolated cells in vitro (Spiliotis et al. 2008). This cyst-like structure seems to be a model of vesicular protoscoleces, a state acquired in secondary hosts, not a consequence of posterior bladder formation, found in vitro in degenerate *pe.* (Heath and Osborn 1976) due to the presence of moving

cells inside the cyst. These structures did not fail to appear in a more or less concentrated agarose, which could indicate a physical effect on isolated cells that allows structure development. Perhaps this could be explained by taking into account the extracellular matrix water content and elasticity, which may lead to differences in cyst development in different organs or hosts, as has been described for microtriches and cyst surface (Bortoletti and Diaz 1978) area and for hydatid liquid composition (Vidor et al. 1986). Another possibility is that the group of cells forming a syncytium, under specific culture conditions and in a rich reducing and nutrient-poor medium, trigger cyst structure formation, changing cell metabolism in order to economize and concentrate nutrients, generating some sort of cell structure differentiation. This phenomenon has been described for the nematode *C. elegans*, a worm that grows into a syncytium with a "balloon effect." This change in structure geometry is associated with parallel changes in cell membrane amino acid composition during growth since changes in structure demand protein synthesis (Swire et al. 2009).

This study describes a stable cell line obtained from *pe.* of *E. granulosus* (EGPE) maintained 3 yr in culture, forming cyst structures in axenic controlled medium. It also presents species origin characteristics such as DCO1 sequence and *AgB4* expression. The contribution of a well-characterized cellular model for *E. granulosus* may allow future studies to progress in the body of knowledge on this subject, permitting karyotype and chromosome fusion characterization and physiological studies on acellular membranes, on autocrine signals involved in cyst formation in agarose, and, ultimately, on cell growth. Finally, studies on cell behavior following animal infection and in co-culture with mammalian cells are needed to search for the biological contribution of this cell line in order to further understand *E. granulosus* biology and parasite host invasion.

Conclusions

In the present, study we have characterized a monolayer cell line obtained from *E. granulosus* bovine protoscoleces maintained 3 yr in vitro, including growth characteristics, morphology by light, fluorescent and electronic microscopy, and karyotype studies. Cell culture origin was confirmed by immunofluorescent detection of *AgB4* and by PCR for mitochondrial DCO1 gene. We ultimately found that cells seeded in agarose biphasic culture resembled a cystic structure, similar to the one formed in secondary hosts. Moreover, a stable cell line, which forms cysts in the neutral matrix of agarose, could represent a significant further approach toward understanding equinococcal behavior.

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