Veterinary Parasitology 205 (2014) 125–133

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Bovine IgG subclasses and fertility of *Echinococcus granulosus* hydatid cysts

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ARTICLE INFO

Article history:
Received 14 March 2014
Received in revised form 2 June 2014
Accepted 3 June 2014

Keywords:
Echinococcus granulosus
Cyst fertility
IgG subclass

ABSTRACT

Hydatidosis is an important zoonotic disease of worldwide distribution, causing important health problems to humans and major economical losses in infected livestock. *Echinococcus granulosus*, the etiological agent of hydatid disease, induces a humoral immune response in the intermediate host (human and herbivorous) against hydatid cyst antigens. Specifically, IgGs are found in the laminar and germinal layers and inside the lumen of fertile and infertile hydatid cysts. In the germinal layer of infertile cysts IgGs are found in an order of magnitude greater than in the germinal layer of fertile cysts; a fraction of those IgGs are associated with high affinity to germinal layer proteins, suggesting their binding to specific parasite antigens. We have previously shown that those immunoglobulins, bound with high affinity to the germinal layer of hydatid cysts, induce apoptosis leading to cyst infertility.

In the present work the presence of IgG1 and IgG2 subclasses in the germinal layer of both fertile and infertile hydatid cysts is reported. IgG1 is the most relevant immunoglobulin subclass present in the germinal layer of infertile cysts and bound with high affinity to that parasite structure. Contrarily, though the IgG2 subclass was also found in the germinal and adventitial layers, those immunoglobulins show low affinity to parasite antigens.

We propose that the binding of an IgG1 subclass to parasite antigens present in the germinal layer is involved in the mechanism of cyst infertility.

1. Introduction

Hydatidosis or cystic echinococcosis (CE) is a major zoonotic disease caused by the infection of mammals with the metacestode stage (hydatid cyst) of the flatworm *Echinococcus granulosus*. It has a worldwide distribution with an estimated 4 million people infected and another 40 million at risk (Aziz et al., 2011). Additionally, economical losses associated to infected livestock were calculated up to US$2,190,132,464 in 2006, being an important challenge for veterinary research (Battelli, 2009).

The greatest prevalence of cystic echinococcosis in human and animal hosts is found in countries of temperate zones, including several parts of Eurasia (the Mediterranean regions, southern and central parts of Russia, central Asia, China), Australia, some regions of America (especially South America) and north and east Africa (Grosso et al., 2012). Recently, the World Health Organization included

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http://dx.doi.org/10.1016/j.vetpar.2014.06.003
0304-4017/

hydatidosis as part of a Neglected Zoonosis subgroup for its 2008–2015 strategic plans devoted to the control of neglected tropical diseases (Siracusano et al., 2012).

The adult flatworm inhabits the small intestine of a carnivore (definitive host, usually canines) and produces eggs containing infective oncospheres. After oral uptake of eggs by an intermediate host animal (herbivores as well as human), the metacestode or larval stage (hydatid cyst) developing in internal organs (mainly liver and lungs) is a fluid-filled, bladder like, unilocular structure (Hsu et al., 2013). These consist of two parasite derived layers, an inner nucleated germinal layer and an outer acellular laminated layer (da Silva, 2011), surrounded by a host-produced fibrous capsule called adventitial layer (Zhang et al., 2003). Protoscoleces, the developmental forms of the parasite infective to canine, are formed in the germinal layer of hydatid cysts. Depending on growth rate, localization, and number of cysts, the infection may remain asymptomatic or may turn to symptomatic (Eckert et al., 1995). Clinical signs in intermediary hosts derive from the mechanical damage produced by hydatid cysts on target organs (Gottstein and Reichen, 2002; McManus et al., 2003; Sayek et al., 2004). CE is usually asymptomatic for a long period of time, because cyst growth is commonly slow; the most frequent symptoms are fatigue and abdominal pain. Patients may also present jaundice, hepatomegaly or anaphylaxis, due to cyst leakage or rupture (Nunnari et al., 2012). In livestock, particularly cattle and sheep, this illness affects the production of meat and viscera as well as of wool, milk and fertility of infected animals (Battelli, 2009). Prevalence of CE in cattle varies according the geographical distribution of the infected animals. Thus, in Iran the prevalence rate of CE in cattle and calf was 6.5% and 8.2%, respectively (Azami et al., 2013), in Saudi Arabia 3.63% (Ibrahim, 2010), in the central Peruvian Andes 80% (Moro et al., 1997) whereas in Chile was 24% (Acosta-Jamett et al., 2010). Two types of hydatid cysts can be observed in intermediate hosts: fertile cysts, in which protoscoleces are found both joined to the germinal layer and free in the hydatid fluid filling the cyst cavity, and infertile cysts, which do not produce protoscoleces and are therefore unable to continue with the life cycle of the parasite (Daryani et al., 2009; Kamenetzky et al., 2000; Lahmar et al., 2004). The biological and molecular background underlying the generation of both types of cysts have not yet been elucidated (Vatankhah et al., 2003); however previous studies suggests that apoptosis is involved in a negative regulation of protoscoleces generation, leading to hydatid cyst infertility (Paredes et al., 2007). It was also proposed that DNA damage triggers cyst infertility of hydatid cysts as well as protoscoleces death (Cabrera et al., 2008).

There are 10 identified *Echinococcus* sp. distinct genetic types (G1-10) and it has been reported the presence of fertile and infertile cysts produced by the same *E. granulosus* strain (Garippa and Manfredi, 2009; Guarnera et al., 2004; Manterola et al., 2008; Moro and Schantz, 2009). Similarly to CE, prevalence of cattle cyst fertility in slaughtered animals shows variability according the geographical distribution. In Pakistan the cyst fertile rate reported was 75.25% (Latif et al., 2010) while in Iran was 77% (Azami et al., 2013), in Ethiopia 15.9% (Negash et al., 2013) and in Kenia 6.5% (Addy et al., 2012). On the other hand, it has been shown that IgGs present in bovine infertile hydatid cysts are associated with low or with high affinity to the germinal layer, most likely corresponding to non specific and antigen specific antibodies binding, respectively (Paredes et al., 2011).

Usually, helminthic infections induce T lymphocytes activation leading to proliferation and differentiation to effector cells capable of mediating microbicidal activity. Thus, T $CD8^+$ lymphocytes differentiate to cytotoxic T-cells while T $CD4^+$ lymphocytes differentiate to either Th1 or Th2 cells. Susceptibility and resistance to infection are based on the activation of different $CD4^+$ T-cell immune responses. For example, in chronic phases of human CE, elevated serum antibody levels are common, particularly IgG, IgM and IgE, with IgG1 and IgG4 IgG subclasses being predominant (Zhang et al., 2012). In human IgG4 antibody response was associated with cystic development, growth and disease progression, whereas the IgG1, IgG2 and IgG3 responses occurred predominantly when cysts became infiltrated or were destroyed by the human host (Daeki et al., 2000). Otherwise Th1 lymphocytes contribute decisively to the inactive stage of hydatid disease whereas Th2 lymphocytes participate in the active and transitional stages (Rigano et al., 2004).

In this work the presence of bovine specific IgG1 and IgG2 subclasses in the germinal layer of both fertile and infertile hydatid cysts is reported. IgG1 is the most relevant immunoglobulin subclass present in the germinal layer of infertile cysts and bound with high affinity to that parasite structure. Contrarily, though the IgG2 subclass was also found in the germinal and adventitial layer of hydatid cysts, those immunoglobulins show low affinity to parasite antigens.

We propose that intermediary hosts develop different immunological responses against *E. granulosus*, strongly associated to the IgG1/IgG2 dominating response (according to the host immunological capacity), allowing germinal cells of the parasite to generate protoscoleces or not. In bovine the binding of an IgG1 subclass to parasite antigens present in the germinal layer could cause cyst infertility.

2. Materials and methods

2.1. Hydatid cysts

Fresh fertile and infertile *E. granulosus* hydatid cysts were obtained from bovine livers or lungs at abattoirs in Santiago, Chile. Cysts were processed as previously described (Galindo et al., 2003; Martinez et al., 2005). Cysts showing a thin, yellow-brown colour inner surface of the cyst, clear hydatid fluid and absence of protoscoleces either by macroscopic or microscopic inspection were considered infertile. Conversely, fertile cyst presented protoscoleces in the hydatid fluid and a thick, whitish parasite germinal layer. Presence of protoscoleces was confirmed under light microscopy by the appearance of buds, and both grown protoscoleces attached to the germinal layer and free in the hydatid fluid. All animals analyzed present only infertile or fertile cyst and there are no case with fertile and infertile cyst in the same animal. All cysts used, either fertile

or infertile, were at least of 3 cm diameter (Bortoletti and Ferretti, 1978; Galindo et al., 2003; Martinez et al., 2005; Paredes et al., 2007, 2011).

2.2. Inner surface of hydatid cysts

Maintaining aseptic conditions, the hydatid fluid was aseptically aspirated from individual cysts and discarded. Afterwards, the inner surface of fertile and infertile cysts containing the germinal layer was obtained by soft scraping of the inner surface of the cysts (previously washed with 10 ml of sterile phosphate buffered saline (PBS) pH 7.4) (Paredes et al., 2007). The samples were centrifuged at 850 × *g* for 5 min at 4 °C and the supernatant was discarded.

2.3. Protein extraction from the inner surface of hydatid cysts with salt solutions of increasing ionic strength

Proteins from the germinal layer of fertile and infertile cysts were extracted with increasing ionic strength salt solutions as described before (Paredes et al., 2011). Briefly, one gram of germinal layer obtained from at least 20 different fertile or infertile cysts were homogenized in PBS pH 7.2 containing 5 mM PMSF, 2.5 mM Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) and 1 mM EDTA as protease inhibitors using a Potter Elvehjem homogenizer with 50 strokes at 1400 rpm. Aliquots of these homogenates were saved for further analysis. Afterwards, each homogenate was centrifuged at 7000 × *g* for 10 min at 4 °C and the supernatant containing proteins dissolved in PBS (low affinity) were saved. This procedure was repeated until the protein concentration in the supernatant was negligible. All supernatants from the PBS (0.154 M NaCl) extractions were mixed. Proteins were then sequentially extracted from each sediment with 1.0 and 3.0 M NaCl solutions (specific/high affinity bound proteins), as described previously (Paredes et al., 2011). Upon extraction with 1 M NaCl proteins from the inner surface of fertile cysts were completely dissolved. Contrarily, after repeatedly extractions of the inner surface of infertile cysts with 3 M NaCl remaining sediment was obtained. All the extracts and the remaining sediment were saved for further analysis. Protein concentration was measured in each extract using the Bradford assay (Bradford, 1976).

2.4. IgGs isolation by affinity chromatography

Antibodies present in the extracts and in the remaining sediment obtained from the inner surface of fertile or infertile hydatid cysts with increasing ionic strength salt solutions were isolated using the Seize X Protein G Immunoprecipitation Kit (Promega). Briefly, 1 ml of protein G affinity column was packed and equilibrated in 15 ml of binding/wash buffer (0.14 M NaCl pH 7.4, 0.008 M sodium phosphate, 0.002 M potassium phosphate, and 0.01 M KCl). Each sample was mixed with binding/wash buffer and applied to the column. Afterwards, the column was washed with 15 ml of binding/wash buffer and the IgGs were eluted with 5–7 ml of 100 mM glycine pH 2.0. Bradford assays were performed to ensure the efficiency of the procedure and the quantification of the samples (Bradford, 1976; Paredes et al., 2011).

2.5. Immunoglobulins identification by Western blot

Proteins from each extract were separated on SDS-15% polyacrylamide gels in a Mini Protean 3 Electrophoresis chamber (Bio-Rad) under reducing conditions, as described before (Paredes et al., 2011). Electrophoresis was performed at 150 V/gel for 90 min and the separated proteins were identified using the silver staining technique.

Proteins from the extract samples were separated as indicated above, transferred to nitrocellulose paper using a mini Trans-Blot Cell (Bio-Rad), blocked overnight at 4 °C using 0.5% soybean proteins in PBS and washed in PBS/0.005% Tween 20. Blots were then incubated for 1 h at room temperature with HRP conjugated anti bovine IgG1 (#A10-116P, Bethyl Laboratories) or HRP conjugated anti bovine IgG2 (#A10-117P, Bethyl Laboratories) at 1/5000 (v/v) dilution. The conjugates were developed with ECL Western blotting in Hyperfilm (GE Healthcare).

2.6. IgG1 and IgG2 densitometric analysis

SDS-Page and Western blot results were captured and digitalized with Doc-Print II (Vilber Lourmat). Afterward the images were processed with Photo-Capt software (Vilber Lourmat). Then, all Western blots were analyzed with Quantity One Software version 4.6.3 for Windows. General background was reduced from the Western blots; bands corresponding to IgG1 or IgG2 were quantified using a contour tool.

2.7. Quantification of IgG (whole molecule), IgG1 and IgG2 by ELISA

Proteins from the inner surface of fertile and infertile hydatid cysts extracted with 0.154 M, 1.0 M and 3.0 M NaCl were adhered to a 96 wells microplate with Coating buffer (0.5 M Na_2CO_3, 0.35 M $NaHCO_3$, 0.03 M NaN_3) pH 9.6 and incubated overnight at 4 °C. Wells were washed with PBS/0.005% Tween 20 and blocked for 1 h at 37 °C using 1% soybean proteins in PBS/0.05% Tween 20. Then, the samples were washed again in the same solution and incubated for 1 h at 37 °C with HRP conjugated anti bovine IgG whole molecule (Jackson ImmunoResearch N° 301-035-003), or with HRP conjugated anti bovine IgG1 or with HRP conjugated anti bovine IgG2 at 1/7500 (v/v) dilution each and developed with 1-Step Slow TMB – ELISA (Thermo Scientific). Analysis of the colorimetric reactions was performed with the BioTek Synergy MX Multi-Mode Microplate Reader and the Gen 5 version 1.08 for Windows software.

2.8. Immunohistochemistry assay of IgGs (whole molecule), IgG1s and IgG2s in fertile and infertile hydatid cysts

Samples of cyst wall from both fertile and infertile cysts were fixed in Glyofix® (Thermo) and embedded

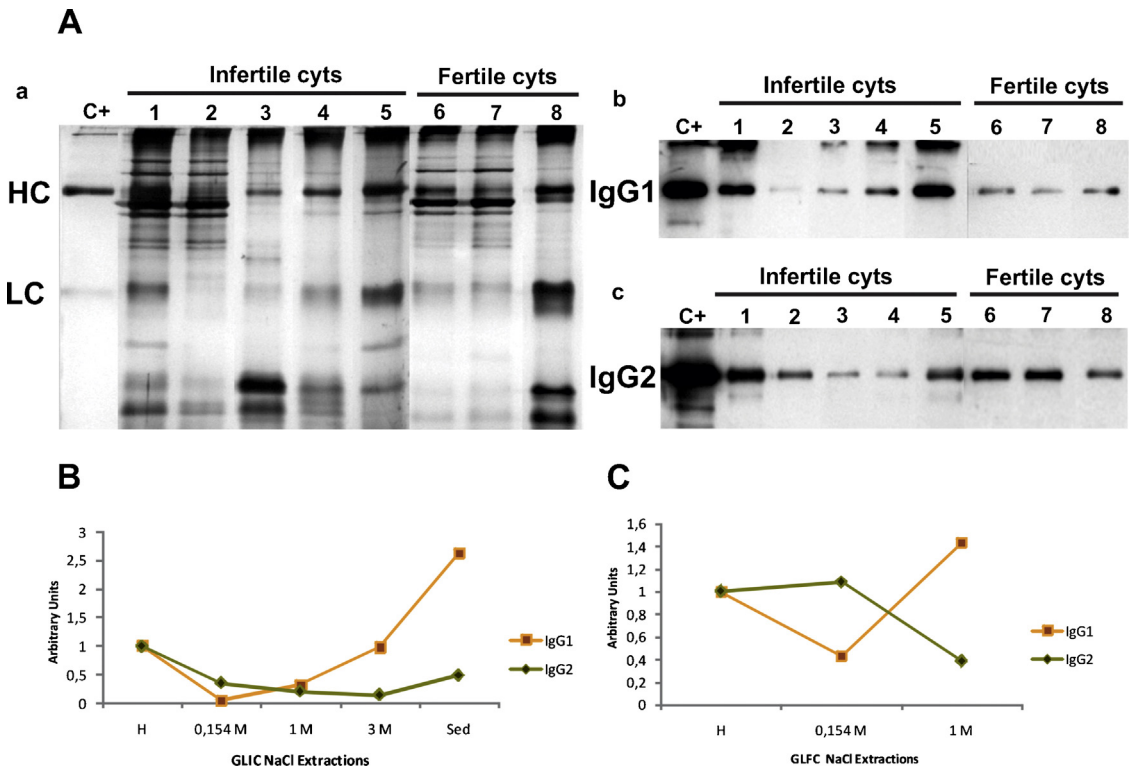

Fig. 1. Bovine IgG1 and IgG2 subclasses are present in the inner surface of fertile and infertile hydatid cysts. (A) Proteins separated in SDS-15% PAGE, 5 μg per lane. (C^+) Heavy (HC) and light (LC) IgG chains from bovine serum. (Lanes 1 and 6) Homogenates from the inner surface of infertile and fertile cysts respectively; (lanes 2 and 7) proteins extracted with 0.154 M NaCl from infertile and fertile cysts, respectively; (lanes 3 and 8) proteins extracted with 1 M NaCl from infertile and fertile cysts, respectively; (lane 4) proteins extracted with 3 M NaCl from infertile cysts; (lane 5) proteins present in the sediment remaining after exhaustive extraction with 3 M NaCl from infertile cysts. (a) Silver stain. (b) Identification of bovine IgG1s by Western blot, using an anti-bovine IgG1 HRP conjugated and developed by chemiluminescence. Bands are depicted as in a. (c) Identification of bovine IgG2 by Western blot, using an anti-bovine IgG2 HRP conjugated and developed by chemiluminescence. Bands are depicted as in a. (B) Densitometric analysis of IgG1 and IgG2 subclasses present in the homogenate (H) and in the extracts obtained with increasing ionic strength salt solutions (0.154 M, 1 M and 3 M NaCl) from the inner surface of infertile hydatid cysts. Sed: Sediment remaining after the 3 M NaCl extraction. (C) Densitometric analysis of IgG1 and IgG2 subclasses present in the homogenate (H) and in the extracts obtained with increasing ionic strength salt solutions (0.154 M and 1 M NaCl) from the inner surface of fertile hydatid cysts. The inner surface of fertile cysts was completely dissolved in 1 M NaCl.

in paraffin. 5 μm sections were placed on Thermo Scientific Superfrost® Plus microscope slides, dewaxed in xylane and intrinsic peroxidases were blocked with 0.9% H_2O_2 in Methanol. Samples were washed with PBS and blocked overnight with ScyTek Pro-Block. Both fertile and infertile cyst wall samples were then incubated with either 1:1000 (v/v) Jackson ImmunoResearch Peroxidase-conjugated AffiniPure Rabbit Anti-Bovine IgG (H + L), 1:500 (v/v) Bethyl HRP conjugated Sheep Anti-Bovine IgG1 or 1:500 (v/v) Bethyl HRP conjugated Sheep Anti-Bovine IgG2. Slides were washed with PBS 1×, developed with Vector ImmPACT™ DAB and counterstained with haematoxylin. Digital images were obtained using an Olympus BX 41 Microscope connected to a Micropublisher 3.3 RTV camera.

3. Results

IgGs subclasses 1 and 2 are present in the inner surface of both fertile and infertile hydatid cysts. Fig. 1A, a shows the protein pattern obtained by SDS-PAGE from the inner surface homogenates and extracts with salt solutions of increasing ionic strength from infertile (Fig. 1A, a, lanes 1–5) and fertile (Fig. 1A, a, lanes 6–8) hydatid cysts. Bands showing electrophoretic mobility similar to heavy (HC) and light (LC) bovine IgG chains (Fig. 1A, a, lane C^+) are present in all preparations analyzed though are more evident in the sediment remaining after 3.0 M NaCl extraction from the inner surface of infertile cysts and in the 1.0 M NaCl extract from the inner surface of fertile cysts (Fig. 1A, a, lanes 5 and 8, respectively).

Similarly, IgG1 (Fig. 1A, b) and IgG2 (Fig. 1A, c) subclasses were clearly evidenced by Western blot analysis in homogenates and in all salt extracts from the inner surface of both infertile (lanes 1–5) and fertile (lanes 6–8) cysts. Densitometric analysis of IgG1s found in the inner surface of infertile cysts (Fig. 1B, squares) shows that the relative concentration of IgG1s regarding the one in the homogenate decreases in the 0.154 M and in the 1 M NaCl extracts, returning to initial levels in the 3 M NaCl extract and increasing 2.6-fold in the remaining sediment after extraction with 3 M NaCl. Contrarily, the concentration of IgG2s relative to the one found in the homogenate (Fig. 1B, diamond) decreases and remains low in all NaCl extracts and in the remaining sediment as well.

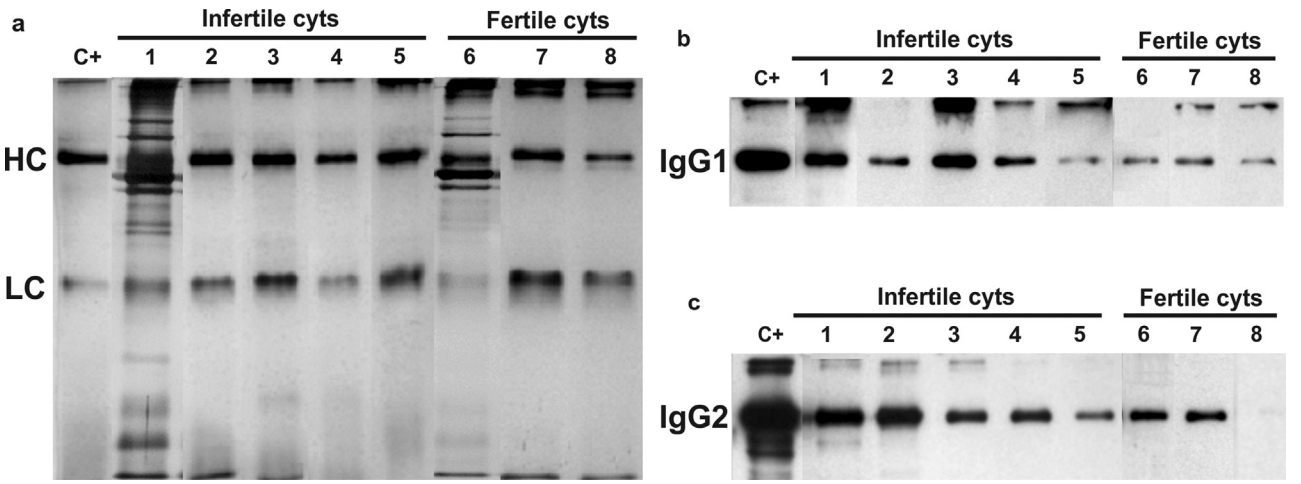

Fig. 2. Bovine IgG1 and IgG2 present in total IgGs purified from proteins extracted from the inner surface of infertile and fertile hydatid cysts. (a) Heavy (HC) and light (LC) chains from bovine IgGs separated in SDS-15% PAGE, 500 ng per line, silver stain. (Lanes 1 and 6) Homogenates from the inner surface of infertile and fertile cysts, respectively; (lanes 2 and 7) bovine IgGs isolated from proteins extracted with 0.154 M NaCl from infertile and fertile cysts, respectively; (lanes 3 and 8) bovine IgGs isolated from proteins extracted with 1 M NaCl from infertile and fertile cysts, respectively; (lane 4) bovine IgGs isolated from proteins extracted with 3 M NaCl from infertile hydatid cysts; (lane 5) bovine IgGs isolated from proteins present in the sediment remaining after exhaustive extraction with 3 M NaCl from infertile cysts. (b) Identification of bovine IgG1 by Western blot, using an Ab anti-bovine IgG1 HRP conjugated and developed by chemiluminescence. Bands are as depicted in a. (c) Identification of bovine IgG2 by Western blot, using an Ab anti-bovine IgG2 HRP conjugated and developed by chemiluminescence. Bands are depicted as in a.

On the other hand, densitometric analysis of IgG1s from the inner surface of fertile cysts (Fig. 1C, squares) shows a decrease in the 0.154 M extract regarding the one found in the homogenates while increasing in the 1.0 M NaCl extract. Contrarily, the IgG2s proportion in the 0.154 M extract remains similar to the one found in the homogenate while decreasing in the 1 M extract. Taking into consideration that IgGs are removed from parasite antigens present in the hydatid cyst inner surface at salt concentrations of 1.0 M NaCl or higher (Paredes et al., 2011), IgG1s subclass appears as the immunoglobulin subclass strongly and specifically bound to parasite antigens, particularly in the germinal layer of infertile cysts (Fig. 1B). This result implies that the bovine host elicits a humoral response against parasite antigens present in hydatid cysts evidenced by specific IgG1s mainly found in the germinal layer of infertile cysts.

To better test this finding IgGs subclasses 1 and 2 were assayed in total IgGs purified from the inner surface of both fertile and infertile hydatid cysts.

Fig. 2a, lane C^+ shows IgGs heavy (HC) and light (LC) chains isolated from bovine serum. In lanes 1 and 6 the protein pattern obtained by SDS-PAGE from the inner surface of infertile and fertile cysts homogenates, respectively, is shown. Lanes 2–5 show the HC and LC of IgGs isolated from the 0.154 M (lane 2), 1.0 M (lane 3) and 3.0 M NaCl (lane 4) as well as from the remaining sediment after the 3.0 M NaCl extraction (lane 5) of the inner surface of infertile cysts. Lanes 7 and 8 show the HC and LC of IgGs isolated from the 0.154 M (lane 7) and 1.0 M (lane 8) extracts of the inner surface of fertile cysts. Clearly, HC and LC from isolated IgGs are present in all saline extracts and in both, infertile and fertile hydatid cysts inner surfaces.

Using specific antibodies, IgG1 (Fig. 2b) and IgG2 (Fig. 2c) subclasses were evidenced by Western blot analysis in inner surface homogenates from infertile and fertile hydatid cysts. However, the IgG1 subclass was predominant in the infertile cyst germinal layer (compare Fig. 2b, lanes 1–5 with lanes 6–8) while the IgG2 subclass levels remain constant in all fractions (Fig. 2c). The signal for the IgG2 subclass was extremely weak in the 1.0 M NaCl extract from the fertile cysts (Fig. 2c, lane 8) whereas the IgG1 subclass band was strong in the 1.0 M extract from infertile cysts (Fig. 2b, lane 3) and still clearly present in the 3.0 M extract (Fig. 2b, lane 4) as well as in its remaining sediment (Fig. 2b, lane 5). Again, these results point to an association of the IgG1 subclass to cyst infertility.

To assay the relative proportion of each IgG subclass relative to total IgGs extracted and purified from the inner surfaces of both, infertile and fertile hydatid cysts, ELISA analysis were performed in total homogenates and in each salt extract using specific antibodies.

Clearly, the IgG1 proportion in the homogenate and in all salt fractions is higher than the observed for IgG2, in infertile as well as in fertile cysts (Fig. 3A and B). Though the proportion of IgG2s in the 0.154 M NaCl extract is of relative importance, both in infertile and fertile cysts, the fact that this subclass is easily extracted with solutions of low salt concentration indicates that it is only weakly bound to parasite proteins. Contrarily, IgG1s are tightly bound to parasite proteins.

In order to localize the IgG1 and IgG2 subclasses in the adventitious, laminar and germinal layers of bovine infertile and fertile cysts, an immunocytochemistry analysis was performed. Histological sections from fertile and infertile cyst walls stained with HE are shown in Fig. 4a and e, respectively. As expected, fertile cysts showed buds and

A

		IgG1/H+L	IgG2/H+L
GLIC	H	0,90	0,16
	0,154 M	0,93	0,60
	1 M	1,03	0,13
	3 M	1,08	0,16
	Sed	1,04	0,13
GLFC	H	0,88	0,17
	0,154 M	0,92	0,30
	1M	0,98	0,11

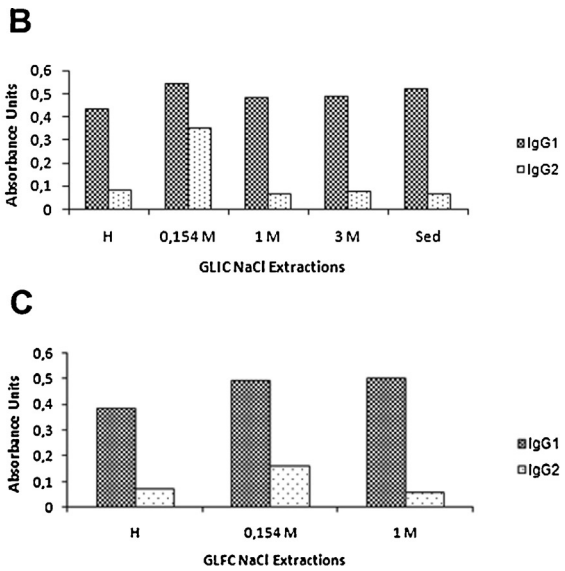

Fig. 3. ELISA analysis of IgG subclasses present in protein extractions with increasing ionic strength salt solutions from the inner surface of fertile and infertile hydatid cysts. GLIC: germinal layer of infertile hydatid cysts. GLFC: germinal layer of fertile hydatid cysts H: homogenate from the inner surface of hydatid cysts; 0.154 M, 1 M and 3 M: Proteins extracted with 0.154, 1.0 or 3.0 M NaCl, respectively from the inner surface of hydatid cysts. (A) Comparative analysis between IgG subclasses (IgG1 and IgG2) and IgG whole molecule (H + L), displayed in a porcentual table of bovine IgG ELISA. (B) Graphical representation of IgG1/IgG2 ratio present in germinal layer of infertile hydatid cysts. (C) Graphical representation of IgG1/IgG2 ratio present in germinal layer of fertile hydatid cysts.

brood capsules of fertile cysts with protoescoleces emerging from the germinal layer (Fig. 4a) which were absent in infertile cysts (Fig. 4e). Total bovine IgGs and well as IgG1 and IgG2 subclasses were detected in hydatid cysts sections using specific antibodies, as indicated under Materials and Methods. A faint IgG signal (brown colour) was recorded in the germinal layer of fertile and brood capsules of fertile cysts but not in protoscoleces (Fig. 4b). Contrarily, a strong reactivity for bovine immunoglobulins was present at the germinal layer of infertile cysts (Fig. 4f, GL). A clear though more diffuse signal was also observed in the adventitious layer of those cysts (Fig. 4f, AL). Regarding the IgG1 subclass a faint reactivity was observed in the germinal layer of fertile cysts being this signal more evident at the laminated layer (Fig. 4c). Contrarily, infertile cysts showed a strong reactivity for IgG1, concentrated at the germinal

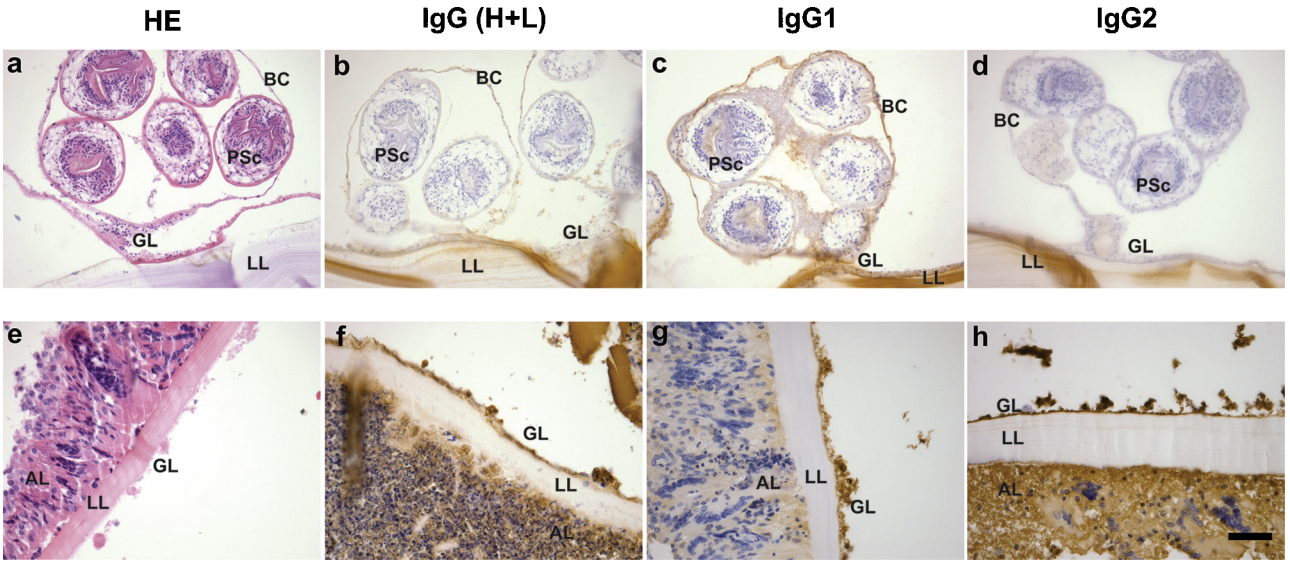

Fig. 4. Detection of bovine IgG whole molecule, IgG1 and IgG2 immunoglobulins by immunohistochemistry in fertile and infertile cysts. (a and e) HE stained fertile and infertile cysts, respectively; (b and f) IgG (whole molecule) in fertile and infertile cysts, respectively; (c and g) bovine IgG1 subclass in fertile and infertile cysts, respectively; (d and h) bovine IgG2 subclass in fertile and infertile cysts, respectively. BC: brood capsules; GL: germinal layer; LL: laminated layer; AL: adventitious layer. Size bar: 50 μm.

layer (Fig. 4g, GL); this IgG subclass is barely detected in the adventitious layer of infertile cysts (Fig. 4g, AL). The IgG2 subclass was present in the laminated layer (LL) of fertile cysts only; no signal was detected at the germinal layer (GL) of cysts and as well as brood capsules (Fig. 4d). However, similarly to total IgGs detection (Fig. 4f) the IgG2 subclass was present in both the adventious (AL) and germinal (GL) layers of infertile cysts. Thus, IgG1 was the bovine IgG subclass mostly associated to the germinal layer of hydatid cysts and concentrated in that structure of infertile cysts.

4. Discussion

Previous studies have demonstrated that infection with helminths produce activation of T lymphocytes. Thus, some parasites have evolved the capacity of inducing host Th2 responses that protect themselves against a potentially toxic anti-parasitic Th1 response (Infante-Duarte and Kamradt, 1999; MacDonald et al., 2002; Rigano et al., 2007; Xu et al., 2010). *E. granulosus* induces a balance between the immune Th1 and Th2 host responses. In intermediate hosts, early infections with *E. granulosus* are characterized by a cellular immune response and, at later stages, by a Th2 type response (Cardozo et al., 2002; Figueiredo et al., 2010). Both humoral and T-cell mediated responses seem to play an important role against hydatid cyst infection. In human, Th1 cells produce interferon-γ (IFNγ), IL-2 and tumour necrosis factor-β (TNF-β), that are responsible for both humoral and cell mediated immune responses. On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 cytokines, which facilitate the production of IgE, IgG1 and IgG4 subclasses (Chandrasekhar and Parija, 2009; MacDonald et al., 2002). In chronic infections with *E. granulosus* high levels of serum IgG, IgM, and IgE have been found (Zhang et al., 2003). In human, the Th2 response is expressed by the appearance of a predominant IgG4 subclass which is associated with progressive hydatid cysts. Conversely, Th1 cell activation is assumed to induce a protective immunity (Chandrasekhar and Parija, 2009; Ortona et al., 2003). Studies designed to assess the IgG-subclasses response in human sera have shown that the most important antibody subclass in both cystic and alveolar echinococcosis is IgG1 (that correlates with inactive disease) and IgG4 (that correlates with active disease) (Chandrasekhar and Parija, 2009; Delunardo et al., 2010). At present, IgGs subclasses have not been directly assayed in hydatid cysts.

Studies in cattle have shown that in most cases of progressive hydatid cysts, $CD8^+$ lymphocytes predominate in the periparasitic region, whereas $CD4^+$ lymphocytes predominate in the adventitial layer surrounding the regressive hydatid cysts. This last observation is associated with an abundant eosinophilic infiltration of the hydatid cyst adventitial layer, probably related with the destruction of the laminar layer (Amri et al., 2009; Ortona et al., 2003; Paredes et al., 2011; Sakamoto and Cabrera, 2003).

With the finding of bovine immunoglobulins, specifically IgGs, in the hydatid fluid present in the lumen of the cyst, it was suggested that those antibodies have the capacity to penetrate the laminar and germinal layers of hydatid cysts (Coltorti and Varela-Diaz, 1972, 1975; Shapiro et al., 1992; Varela-Diaz and Coltorti, 1972). Considering that the protoscoleces are formed from the germinal layer of hydatid cysts (Galindo et al., 2002, 2008; Martinez et al., 2005) the localization of whole IgGs in hydatid cysts and their effects on their survival was recently studied (Paredes et al., 2011). A higher concentration of bovine IgGs in the inner surface of infertile hydatid cysts than in the germinal layer of fertile cysts was found, being the affinity of those IgGs to that cyst structure compatible with an antigen–antibody specific binding. Contrarily, the germinal layer of fertile cysts presents IgGs bound with low affinity to that parasite structure. Consequently, it was proposed that IgGs specific to parasite antigens located at the germinal layer induced cyst infertility (Paredes et al., 2011).

In this work, we have found IgG1 and IgG2 subclasses in the germinal layer of both fertile and infertile hydatid cysts. IgG1 is the most relevant immunoglobulin subclass present in the germinal layer of infertile cysts and bound with high affinity to that parasite structure. Contrarily, though the IgG2 subclass was also found in the germinal and adventitial layers, those immunoglobulins show low affinity to parasite antigens.

We have previously found that IgGs, bound with high affinity to the germinal layer of infertile cysts and extracted with salt solutions of high ionic strength, are recognized by up to five parasite proteins from protoescoleces while IgGs extracted from the germinal layer of fertile cysts with salt solutions of low ionic strength did not recognize any parasite protein (Paredes et al., 2011). We have also found that IgGs bound with high affinity to the inner surface of hydatid cysts induce apoptosis leading to cyst infertility and/or alter and block cellular differentiation processes necessary for protoscolex formation (Galindo et al., 2002; Martinez et al., 2005; Paredes et al., 2007).

Taking into consideration the location in the cyst structure and the affinity to parasite antigens at the germinal layer, we now propose that a humoral immune response of the host against the parasite larval form, which is represented by IgGs, is able to cross the tegument and plasma membrane present between the laminar and the germinal layers of hydatid cysts, resulting in the binding of an IgG1 subclass to parasite antigens leading to the infertility of the hydatid cyst. We are now in the process of identifying the specific parasite antigens which are recognized by the IgG1.

Conflict of interest statement

We declare that there are no conflicts of interest.

Acknowledgements

This study was funded by FONDECYT-Chile N° 1130717. We would like to thank the veterinarians from La Pintana slaughterhouse in Santiago, Chile for their help in sampling the animals used in this study.

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