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Recent Advances in the Immunology and Serological Diagnosis of Echinococcosis

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1. Introduction

Echinococcosis is a near-cosmopolitan zoonosis caused by tapeworms (cestodes) belonging to the family Taeniidae and the genus *Echinococcus*. This parasitic disease is very common but largely neglected. The two major species of medical and public health importance are *Echinococcus granulosus* (Eg) and *E. multilocularis* (Em), which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. Globally, CE is responsible for most of the burden of echinococcosis (Budke, 2006), although AE is re-emerging with increasing frequency in Europe (Deplazes, 2006; Eckert *et al.*, 2000; Romig, 2009; Hegglin *et al.*, 2008).

There are 4 million people infected and more than 100 million at risk of infection including many in the EU countries (Craig and Larrieu, 2006; Craig *et al.*, 2007; McManus *et al.*, 2003). In the endemic regions, human incidence rates can reach more than 50 per 100 000 person/year and prevalence as high as 5–10% may occur, as in parts of Peru, Argentina, east Africa and central Asia (Altintas, 2003; Craig *et al.*, 2007; Moro *et al.*, 1997). In China, there are 60 million people at risk (Ito *et al.*, 2003b) with 550,000 CE patients having visible hydatid cysts by ultrasound (Collaboration projects, 2005; Li *et al.*, 2005) and > 100 million animals infected (Chi *et al.*, 1989).

2. Life-cycle and Echinococcosis

The complex life cycle of *Echinococcus* involves two hosts (Fig 1); a definitive host and an intermediate host. Definitive hosts are carnivores such as dogs, wolves and foxes. Sexual maturity of adult *E. granulosus* occurs in the host small intestine within 4 to 5 weeks of

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ingesting offal containing viable protoscoleces. Gravid proglottids or released eggs are shed in the feces.

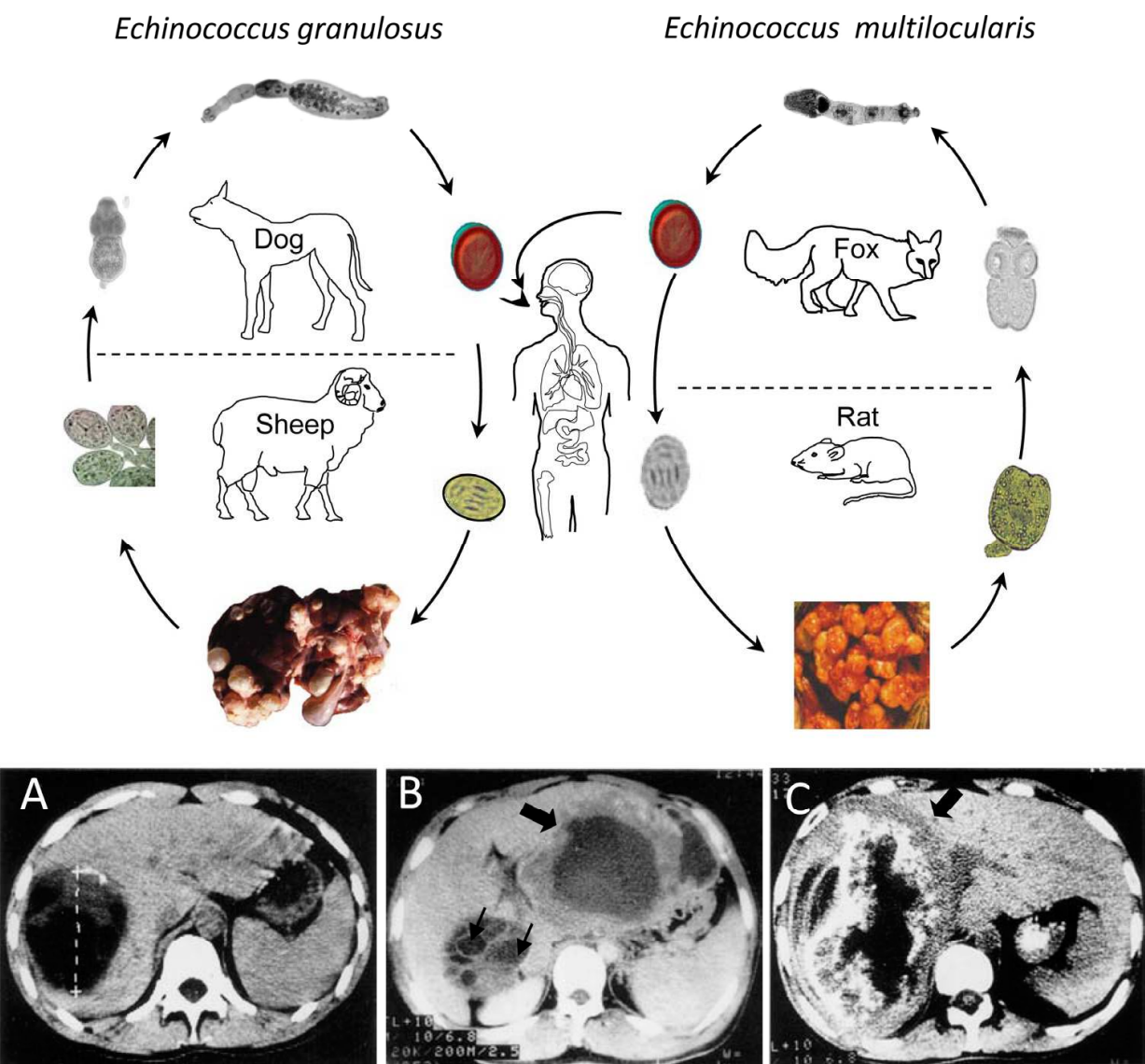


Fig. 1. Life cycle of *Echinococcus* (upper panel) and hepatic echinococcosis scanned by computed axial tomography (CT) (lower panels). A, Cystic echinococcosis (CE) due to *E. granulosus*. B, a patient infected with both CE (daughter cysts are marked with small arrowheads) and alveolar echinococcosis (AE, large arrowhead). C, AE due to *E. multilocularis* infection (arrowhead).

Intermediate hosts are herbivores such as sheep, horses, cattle, pigs, goats, camels, moose, kangaroos and wallabies. Humans are accidental hosts and generally play no part in transmission. Intermediate hosts and humans become infected by ingesting eggs of the parasites, which are released in the faeces of the definitive hosts. The eggs hatch in the gastrointestinal tract and become activated larvae (oncospheres) which penetrate the intestinal wall and enter the bloodstream, eventually locating in internal organs, where they develop into hydatid cysts. Hydatid cysts of *E. granulosus* develop in internal organs of intermediate hosts and humans as fluid-filled bladders (Figure 1A). The cysts can be located

in almost all organs, with about 70% of cysts in the liver, 20% in the lungs, with the remainder involving other organs such as the kidney, spleen, brain, heart and bone.

A typical cyst of *E. granulosus* consists of two parasite-derived layers; an inner nucleated germinal layer and an outer acellular laminated layer surrounded by a host-produced fibrous capsule. Brood capsules and protoscoleces bud off from the germinal membrane.

The alveolar cyst or metacestode of *E. multilocularis* develops differently to that of *E. granulosus*, being a complex tumor-like multivesicular, infiltrating structure consisting of numerous small vesicles embedded in stroma of connective tissue (Fig 1B and C). The larval mass usually contains a semisolid matrix, rather than fluid, with granulomatous infiltration of mononuclear cells around the parasitic vesicles, a hallmark of AE, culminating in irreversible fibrosis. Adult worm infections of *E. multilocularis* occur mainly in red and arctic foxes, although dogs and cats (rarely) can also act as definitive hosts. Small mammals (usually microtine and arvicolid rodents) act as intermediate hosts (Fig 1).

The chronic disease of CE is characterized in humans and domestic and wild ungulates by long term growth of metacestode (hydatid) cysts in the internal organs of intermediate hosts. Clinical manifestations are mild in the early stages of infection, and normally remain asymptomatic for a long period, but the host does produce detectable humoral and cellular responses against the infection, while the parasite has evolved highly effective evasive strategies to aid in long term survival. Immunity to *E. granulosus* infection is typical of a chronic infection and the host responses are pivotal for developing laboratory-based diagnostic procedures.

The proliferative larval stages of *E. granulosus* and *E. multilocularis* can 'leak' out of a ruptured cyst (*E. granulosus*) or metastasize (*E. multilocularis*) to another organ or tissue naturally producing a condition known as secondary CE or AE, respectively. This also allows the parasites to be passaged serially from one intermediate host to another by intraperitoneal implantation of the larvae, simplifying the technical difficulties and danger associated with cyclic passage through both definitive and intermediate hosts.

CE and AE are both serious diseases, the latter especially so, with a poor prognosis if careful clinical management is not carried out. In the later stages of echinococcosis, as the disease progresses, the parasite may physically damage tissues and organs which can become dysfunctional and can be fatal, especially in AE, when the parasite destroys the liver parenchyma, bile ducts and blood vessels resulting in biliary obstruction and portal hypertension. In most late-stage cases a necrotic cavity, containing viscous fluid, may form in the liver.

3. Host immune responses against infection

Immune responses play an important role in the host-parasite interplay in echinococcosis. The mammalian host produces immune responses to reject and/or limit the growth of the parasite, whereas, the parasite produces molecules to avoid these immune attacks. One phenomenon of the infection is self-cure, which is commonly observed in sheep (Zhang and Zhao, 1992; Cabrera *et al.*, 2003) and also occurs in humans in hyper-endemic areas (Wang *et al.*, 2006; Moro *et al.*, 2005; Macpherson *et al.*, 2004). Indeed, more than 70% of cysts surgically removed from a large cohort of Chinese patients were shown to be inactive and

calcified (Peng, XY, personal communication), indicating that the number of human self-cure cases can be substantial. However, details of this important, likely immunologically-based process, remain limited. In addition, some intermediate hosts such as cattle produce a high percentage - >90% - of cysts that are infertile in that they do not contain protoscoleces, the developmental form of the parasite infective to the definitive host (Fig. 1), with host species-specific differences in immunity probably the key factor (Zhang *et al.*, 2003a). Similarly in AE, some patients with calcified lesions (Gottstein and Felleisen, 1995) are likely due to self-cure.

This self-cure phenomenon raises the possibility that protective immune responses against parasite growth and dissemination may exist. However, little is known about the determinants which may restrain metacestode survival and proliferation, or eliminate *Echinococcus* infection.

Although the data are limited, there is, nevertheless, clear evidence, from experiments with animals challenged with *E. granulosus*/*E. multilocularis* eggs or oncospheres that infected hosts produce significant immune responses, including antibody and T cell responses generated by lymphocytes. Understanding the mechanisms whereby these immune responses are produced, particularly the role of protective antibodies against the oncosphere, has been of fundamental importance in developing highly effective recombinant vaccines against both *E. granulosus* and *E. multilocularis*.

3.1 Antibody responses

The earliest immunoglobulin (Ig) G response to *E. granulosus* hydatid cyst fluid and oncospherical antigens appears after 2 and 11 weeks, respectively, in mice and sheep challenged with eggs or oncospheres of *E. granulosus* (Zhang *et al.*, 2003b; Yong *et al.*, 1984). Anti-oncospherical antibodies play a major role in parasite killing and are central to the protective immune response against *E. granulosus* (Read *et al.*, 2009). In the chronic phase of CE, elevated antibody levels, particularly IgG, IgM and IgE, occur in humans (Khabiri *et al.*, 2006; Daeki *et al.*, 2000; Pinon *et al.*, 1987; Craig, 1986; Dessaint *et al.*, 1975) (Fig. 2), with IgG1 and IgG4 being predominant (Daeki *et al.*, 2000; Sterla *et al.*, 1999; Wen and Craig, 1994). In murine models of AE infection, IgG and IgM were significantly increased after 9 weeks post-infection (p.i) with eggs (Matsumoto *et al.*, 2010). Antibody responses to protoscolex antigens are relatively weak and delayed in the early stage of *E. multilocularis* infection in mice, but are increased later (Bauder *et al.*, 1999; Matsumoto *et al.*, 1998); IgG1 and IgG3 levels increase significantly at 8 weeks p.i, and remain elevated thereafter (Li *et al.*, 2003a).

Antibody production is a pre-requisite for the development of serodiagnostic tests, but 30-40% of patients are antibody-negative for CE. In many of these patients, however, varying levels of circulating antigens (CAg) and circulating immune complexes (CIC) are measurable (Craig, 1993). This phenomenon suggests that B cell activity and proliferation may be regulated and inhibited by *E. granulosus* antigens. It is not known whether these antigens directly target B cells or via T cell regulatory mechanisms. One study showed that after infection, CD4-knockout mice and C57Bl/6 mice had similar titres of specific antibodies, indicating that antibody production may be T cell-independent in early infection (Baz *et al.*, 2008).

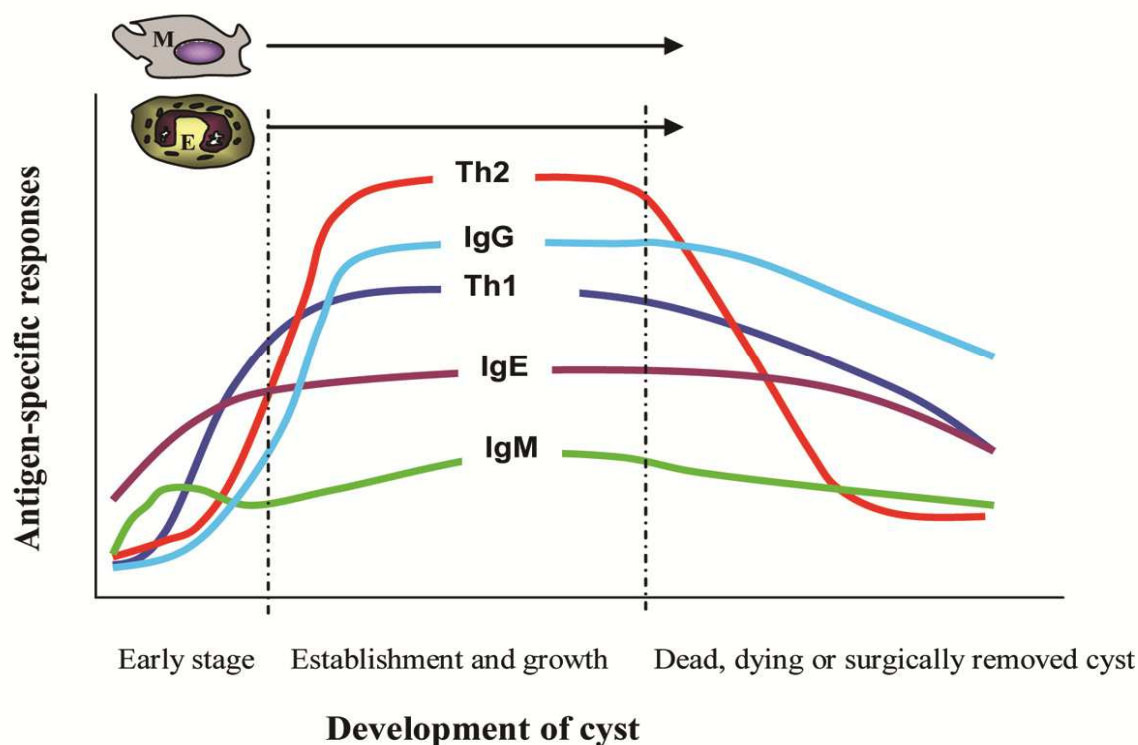


Fig. 2. Immune responses during the development of a hydatid cyst of *E. granulosus* in the intermediate host. In the early stage of infection, the oncosphere is transported to a host organ such as the liver or lung where it develops into a hydatid cyst. The immature cyst has to overcome host, mainly cell-mediated, immune responses, especially the infiltration of macrophages and eosinophils and low level polarized Th1 responses. About 8-10 weeks post-infection in mice, cyst growth is maintained and complex echinococcal antigens are released from the cyst. These antigens stimulate complex immune responses. These include polarized Th2 responses, balanced with Th1 responses. At this time, the parasite produces significant quantities of antigens that help to modulate the immune response, which may benefit both host and parasite; IgG, especially IgG1 and IgG4, IgE and IgM levels are elevated. When the cyst is dead, dying or surgically removed, the Th2 responses drop rapidly, whereas the Th1 responses drop slowly then becoming polarized. IgG can be maintained in the human host for many years after the cyst is surgically removed. Once an infected patient has relapsed, the Th2 responses recover very quickly, while other responses are elevated slowly. M, macrophage; E, eosinophil.

In addition, antibody responses may indicate the infectious status (Li *et al.*, 2010; Delunardo *et al.*, 2010; Reiter-Owona *et al.*, 2009; Ben Nouir *et al.*, 2008; Ortona *et al.*, 2005). A survey of 246 CE patients showed that *E. granulosus* antigen B (AgB) specific antibody was elevated in a significantly greater proportion (87.3%) of subjects with active or transitional stage cysts (CE1, CE2, or CE3), compared with 54.8% of other patients with inactive or early stage cysts (CL, CE4 or CE5). Furthermore, AgB-specific antibody was detected in 95.6% of CE2 cases, which was statistically higher than that (73.7%) in CE1 patients (Li *et al.*, 2010). Results from 173 AE patients showed that serum antibody levels against Em18 were significantly correlated with the disease phase (Li *et al.*, 2010).

A survey of 861 children in China showed that serological tests can also be as an indicator of *Echinococcus* transmission; this study predicated the transmission of echinococcosis due to changes in the ecology and socio-geography in and around endemic communities (Yang *et al.*, 2008).

3.2 T cell responses

During the early stages of an echinococcal infection, there is a marked activation of cell-mediated immunity including cellular inflammatory responses and pathological changes (Zhang *et al.*, 2003a; Zhang *et al.*, 2008b). Cellular infiltration of eosinophils, neutrophils, macrophages, and fibrocytes occurs in humans (Magambo *et al.*, 1995; Peng *et al.*, 2006) and sheep (Petrova, 1968) infections. However, this generally does not result in a severe inflammatory response and aged cysts tend to become surrounded by a fibrous layer that separates the laminated cystic layer from host tissue.

Early Th1 polarized cytokine production, which can kill the metacestode at the initial stages of development (Vuitton, 2003), then shifts to a predominant Th2 cytokine response in the later, chronic stage, and is characteristic of *E. granulosus* and *E. multilocularis* infection (Fig. 2). Very little is known of cytokine production in the early phases of a primary (oral challenge with eggs) *E. granulosus* infection, although both Th1 and Th2 cytokine levels are low in the early stages of a primary *E. multilocularis* infection, but are raised subsequently (Bauder *et al.*, 1999). As well, it is thought the Th2 cytokines are responsible for inhibition of parasite killing because of the anti-inflammatory action of interleukin-10 (IL-10) (Bauder *et al.*, 1999; Vuitton, 2003). The Th2-type cytokines profile in abortive AE patients was opposite to those in progressive patients, with IL -10 and IL-5 associated with the progression of disease (Godot *et al.*, 2000). However, secondary *E. multilocularis* infection in a mouse model showed that Th1 cytokines play a role in resistance of parasite growth (Emery *et al.*, 1997).

Patients with chronic CE generate both Th1 and Th2 responses (Baz *et al.*, 2006). Given the recent advances in understanding the immunoregulatory capabilities of helminthic infections, it has been suggested that Th2 responses play a crucial role in chronic helminthiasis (Allen and Maizels, 2011). However, a remarkable feature of chronic CE infection is the coexistence of interferon-gamma (IFN- γ), IL-4 and IL-10 at high levels in human echinococcosis (Mezioug and Touil-Boukoffa, 2009). It is unclear why hydatid infection can induce high levels of both Th1 and Th2 cytokines (Rigano *et al.*, 1995a) since they usually down-regulate each other (Pearce and MacDonald, 2002). Antigen and the amount of antigens released may play key roles. For instance, *E. granulosus* AgB skewed Th1/Th2 cytokine ratios towards a preferentially immunopathology-associated Th2 polarization, predominantly in patients with progressive disease (Rigano *et al.*, 2001).

Liver pathology in AE is characterized by the presence of a huge granulomatous infiltrate of mononuclear cells involving mainly macrophages, myofibroblasts and T lymphocytes (Harraga *et al.*, 2003; Vuitton *et al.*, 1989). In the progressive forms of the disease, the T cell infiltrate within the periparasitic granuloma is mainly composed of CD8 T lymphocytes (Bresson-Hadni *et al.*, 1990).

A hallmark of chronic *Echinococcus* infection is the presence of high levels of IL-10 (Zhang *et al.*, 2008a; Vuitton, 2003), a cytokine typically associated with immunoregulation of effector responses (Moore *et al.*, 2001). The role of IL-10 in chronic infection largely remains unclear,

although one report showed that IL-10 may impair the Th1 protective response and allow the parasite to survive in hydatid patients (Moore *et al.*, 2001). By inducing the host to produce high levels of IL-10, *E. multilocularis* appears able to modulate the immune response so that the T cells infiltrating the periparasitic granuloma cannot participate in the effector phase of the cellular immune response (Zhang *et al.*, 2008a; Vuitton, 2003). The interaction of the *Echinococcus* organisms with their mammalian hosts may provide a highly suitable model to address some of the fundamental questions remaining such as the molecular basis underpinning the different effects of IL-10 on different cell types; the mechanisms of regulation of IL-10 production; the inhibitory role of IL-10 on monocyte/macrophage and CD4 T cell function; its involvement in stimulating the development of B cells and CD8 T cells; and its role in the differentiation and function of T regulatory cells.

In addition, the production of subgroups of chemokines (CC and CXC) associated with inflammation (MIP-1 alpha (macrophage inflammatory protein 1 alpha)/CCL3, MIP-1 beta/CCL4, RANTES (regulated upon activation, normally T-cell expressed and secreted)/CCL5 and GRO-alpha (growth-regulated oncogene-alpha)/CXCL1) in peripheral blood mononuclear cells stimulated with Em antigens *in vitro* was constitutively larger in AE patients than in controls (Kocherscheidt *et al.*, 2008). However, in patients, Em metacestodes suppressed cellular chemokine production, and this may constitute an immune escape mechanism which reduces inflammatory host responses, prevents tissue destruction and organ damage, but may also facilitate parasite persistence (Kocherscheidt *et al.*, 2008).

It is not known whether the significant cellular infiltration of macrophages and neutrophils occurring as the parasite develops results from the innate immune mechanisms of the host and the release of chemotactic substances by the parasite or whether it is dependent on Th0/Th1 cytokines. A large number of CD4(+) T lymphocytes are present in AE patients with aborted or dead lesions, whereas patients with active parasites display a significant increase in activation of predominantly CD8(+) T cells (Manfras *et al.*, 2002) indicating that CD4(+) T cells may play a role in the killing mechanism. This is supported by experiments undertaken with genetically modified mice (Dai *et al.*, 2001). Conversely, *E. multilocularis* is able to survive and persist in its host indefinitely for long periods of time. In fact, the murine immune response fails to clear infection even when presented with the lowest possible infection dose by injection with a single parasite vesicle (Gottstein *et al.*, 2002).

3.3 Correlation of cytokines with antibody production

It is noteworthy that the increased production of IL-4 and IL-10 in hydatid patients (Bayraktar *et al.*, 2005) corresponds with high levels of IgE and IgG4 (Rigano *et al.*, 1995b), suggesting regulation by IL-4 of IgE and IgG4 responses (Rigano *et al.*, 2001; Rigano *et al.*, 1995b; King and Nutman, 1993). Patients with a primary infection have higher levels of IL-2, IFN- γ and IL-5. The effect of IL-5 on human B cells is controversial (Clutterbuck *et al.*, 1989), but a significant correlation between IL-5 production and IgE/IgG4 expression has been found in hydatid patients (Rigano *et al.*, 1996), indicating that IL-5 is associated with the regulation of specific IgE/IgG4 expression. When CE cysts grow, IgG1 and IgG4 levels are elevated, whereas the concentrations of specific IgG1 and IgG4 decline in cases characterized by cyst infiltration or calcification. This indicates that the IgG4 antibody

response is also associated with cystic development, growth and disease progression, whereas the IgG1, IgG2 and IgG3 responses occur predominantly when cysts become infiltrated or are destroyed by the host (Daeki *et al.*, 2000).

3.4 T cell responses and treatment

The polarized T cell responses in echinococcosis have been shown to be modulated by the developmental status of the hydatid cyst, as shown by experiments with T-cell lines generated from patients with active, transitional and inactive hydatid cysts and stimulated with sheep hydatid fluid antigens. It is likely due to the antigens containing distinct epitopes for each T-cell subset as a single recombinant protein can stimulate both types of response (Zhang *et al.*, 2003a). T-cell lines from a patient with an inactive cyst had a Th1 profile, while T-cell lines derived from patients with active and transitional hydatid cysts had mixed Th1/Th2 and Th0 clones (Rigano *et al.*, 2004), indicating that Th1 lymphocytes contribute significantly to the inactive stage of hydatid disease, with Th2 lymphocytes being more important in the active and transitional stages. When CE patients were drug-treated with albendazole/mebendazole, a Th1 cytokine profile, rather than a Th2 profile, typically dominated, indicating that Th1 responses have a role in the process of cyst degeneration (Rigano *et al.*, 1995b). An increased Th1-type cytokine IFN- γ response has been suggested as a marker for monitoring AE patient treatment (Dvoroznakova *et al.*, 2004), whereas as measurement of serum IL-4 may be a useful marker for the follow up of patients with CE (Rigano *et al.*, 1999b).

One aspect that is likely to be important in the control of such immunological mechanisms is the influence of CD4⁺ T-helper lymphocytes as they may impact on treatment of echinococcosis (Vuitton, 2004). As indicated earlier, self-cure is a common feature of CE infection in sheep (Zhang and Zhao, 1992; Cabrera *et al.*, 2003), and it most likely also happens in human populations in hyper-endemic areas as patients with calcified cysts are often reported (Moro *et al.*, 2005; Macpherson *et al.*, 2004). Cytokines are likely to play a key role in the process of self-cure and this is an important area that needs further research.

Both *in vitro* and *in vivo* studies have shown that high levels of the Th1 cytokine IFN- γ were found in patients who responded to chemotherapy, whereas high levels of Th2 cytokines 1 (IL-4 and IL-10) occurred in patients who did not (Rigano *et al.*, 1999b; Rigano *et al.*, 1999a; Rigano *et al.*, 1995b; Rigano *et al.*, 1995a).

3.5 Dendritic cells

Some recent studies have focused on dendritic cells (DC) and their regulation on other immune responses in CE. *E. granulosus* antigens influence maturation and differentiation of DC stimulated with lipopolysaccharide (LPS) (Kanan and Chain, 2006). This includes down-modulation of CD1a expression and up-regulation of CD86 expression, a lower percentage of CD83(+) cells being present, and down-regulation of interleukin-12p70 (IL-12p70) and TNF alpha (Rigano *et al.*, 2007). In addition, hydatid cyst fluid (HCF) modulates the transition of human monocytes to DC, impairs secretion of IL-12, IL-6 or PGE2 in response to LPS stimulation, and modulates the phenotype of cells generated during culture, resulting in increased CD14 expression (Kanan and Chain, 2006). AgB has been shown to induce IL-1 receptor-associated kinase phosphorylation and activate nuclear factor-kappa B,

suggesting that Toll-like receptors could participate in *E. granulosus*-stimulated DC maturation (Rigano *et al.*, 2007).

E. multilocularis infection in mice induced DC expressing high levels TGF and very low levels of IL-10 and IL-12, and the expression of the surface markers CD80, CD86 and CD40 was down-regulated (Mejri *et al.*, 2011a; Mejri *et al.*, 2011b). However, the higher level of IL-4 than IFN- γ /IL-2 mRNA-expression in AE-CD4⁺ pT cells indicated DC play a role in the generation of a regulatory immune response (Mejri *et al.*, 2011a).

Different *E. multilocularis* antigens have been shown to stimulate different expression profiles of DC. Em14-3-3-antigen induced CD80, CD86 and MHC class II surface expression, but Em2(G11) failed to do so. Similarly, LPS and Em14-3-3 yielded elevated IL-12, TNF-I⁺/⁻ and IL-10 expression levels, while Em2(G11) did not. The proliferation of bone marrow DC isolated from AE-diseased mice was abrogated (Margos *et al.*, 2011), indicating the *E. multilocularis* infection inhibited T cell responses.

3.6 Regulatory T cells

Chronic *Echinococcus* infection results in the suppression of host immune responses, allowing long-term parasite survival and restricting pathology. Current theories suggest that regulatory T cells (Treg) play an important role in this regulation. However, the mechanism of Treg induction during *Echinococcus* infection is still unknown, although several studies have focused on this area.

A subpopulation of regulatory CD4⁺ CD25⁺ T cells isolated from *E. multilocularis*-infected mice reduced ConA-driven proliferation of CD4⁺ pT cells. The high expression levels of Foxp3 mRNA by CD4⁺ and CD8⁺ peritoneal T cells suggested that subpopulations of regulatory CD4⁺ Foxp3⁺ and CD8⁺ Foxp3⁺ T cells were involved in modulating the immune responses in the chronic stage of AE, which are Th2 polarized responses (Mejri *et al.*, 2011b).

A primary infection with oncospheres of *E. granulosus* in mice (Zhang *et al.*, 2001) generated a low level antibody response during the first 8 weeks (Zhang *et al.*, 2003b), possibly indicating host immunosuppression. After 9 weeks of the primary infection, antibodies against hydatid cyst fluid and oncosphere antigens were significantly increased, suggesting that antibody production during the course of the infection may be regulated, perhaps through periodic release of antigen from cysts and/or general down regulation of B-cells through T-helper cell activity.

AgB is the most evaluated echinococcal antigen for its role in modulation of immune responses. AgB significantly inhibits polymorphonuclear cell recruitment (Rigano *et al.*, 2001; Shepherd *et al.*, 1991), modulates dendritic cell differentiation and polarizes immature DC maturation towards a Th2 cell response (Rigano *et al.*, 2007). After establishment in the host, the hydatid cyst produces a large amount of AgB (Zhang *et al.*, 2003b), which can alter the Th1/Th2 ratio from a predominant Th1 response in the early stage of infection to a Th2 response (Rigano *et al.*, 2001), indicating that AgB is a modulator of the T cell response benefiting parasite survival. A number of studies have shown that AgB is encoded by a gene family and the antigen exhibits a high degree of

genetic polymorphism (Haag *et al.*, 2006; Rosenzvit *et al.*, 2006; Kamenetzky *et al.*, 2005), suggesting that the *Echinococcus* organisms have evolved antigenic variation-type mechanisms for escaping the host immune response.

4. Serological diagnosis of human echinococcosis

Typical asymptomatic features in the early stages of infection and for a long period after establishment makes early diagnosis of echinococcosis in humans difficult. Although the definitive diagnosis for most human cases of CE is by physical imaging methods, such as X-ray, ultrasonography, computed axial tomography (CT scanning) and magnetic resonance imaging, these procedures are often not readily available in isolated communities and usually they provide effective diagnosis mainly of the late development stages of clinical infection. Early diagnosis of CE and AE by serology may, therefore, provide opportunities for early treatment and more effective chemotherapy. Another practical application of serology in human echinococcosis is the follow-up of the treatment. Although hydatid disease is an asymptomatic infection, the host does produce detectable humoral and cellular responses against the infection. Measurement of these responses is a prerequisite for developing effective serodiagnostic tools.

4.1 Methodology in detecting antibodies

Almost all serological tests have been developed for immunodiagnosis of human CE cases. These include indirect haemagglutination (IHA), latex agglutination (LA), complement fixation test (CFT), immunoelectrophoresis (IEP) tests and enzyme-linked immunosorbent assay (ELISA) (Rickard *et al.*, 1984; Rickard, 1984). In contrast, the CFT and LA tests are more likely to give non-specific or false positive reactions (Rickard, 1984) as is the IHA test when serum titres are lower than 1:512 (Varela-Diaz *et al.*, 1975c; Varela-Diaz *et al.*, 1975b; Varela-Diaz *et al.*, 1975a; Craig and Nelson, 1984).

There are considerable differences between the various tests both in specificity and sensitivity. As the sensitivity of a test increases, so generally does the demand for improved antigens in order that sufficient specificity can be achieved to take advantage of the greater sensitivity. An optimum test should be specific with high sensitivity. Insensitive and non-specific tests including the Cassoni intradermal test, CFT, IHA and LA test have been replaced by ELISA, IEP, indirect immunofluorescence antibody test (IFAT), and immunoblotting (IB) in routine laboratory application (Lightowers and Gottstein, 1995; Nasrieh and Abdel-Hafez, 2004)). ELISA achieved sensitivity rates of 88 to 98% using cyst fluid preparations (Rickard *et al.*, 1984; Speiser, 1980).

Recently developed dipstick assays (van Doorn *et al.*, 2007) are considered to be valuable methods for CE serodiagnosis. A dipstick assay has been developed that exhibited 100% sensitivity and 91.4% specificity with 26 CE sera and 35 other parasite infection sera using camel hydatid cyst fluid as antigen (Al-Sherbiny *et al.*, 2004). Since the dipstick assay is extremely easy to perform with a visually interpreted result within 15 min, in addition to being both sensitive and specific, the test could be an acceptable alternative for use in clinical laboratories lacking specialized equipment and the technological expertise needed for IB or ELISA.

One study (Ortona *et al.*, 2000) highlights the need to standardize techniques and antigenic preparations and to improve the performance of immunodiagnosis by characterizing new antigens and detecting distinct immunoglobulin classes. The diagnostic sensitivity and specificity of IEP, ELISA and IB, in detecting IgG antibodies in patient sera to native and recombinant AgB and a hydatid fluid fraction (HFF) were compared. Sera tested were from patients who had CE grouped according to their type of cysts, from patients with other parasitic diseases, lung or liver carcinomas or serous cysts, and from healthy controls. HFF-IB gave the highest sensitivity (80%) followed by ELISA (72%) and IEP (31%). The diagnostic sensitivity significantly decreased as cysts matured (from type I-II to type VII, classified by ultrasound). Recombinant and native AgB-IB yielded similar sensitivity (74%) but a large number of clinically or surgically confirmed CE patients (20%) were negative. In these patient sera, IB to assess the usefulness of another recombinant *E. granulosus* molecule (elongation factor-1 beta/delta) in detecting IgE antibodies, yielded 33% of positivity.

The results of this and other studies suggest that hydatid serology may be improved by combining several defined antigens (including synthetic peptides), and the design of new *E. granulosus*-specific peptides that react with otherwise false-negative sera.

4.2 Immunodiagnosis of cystic echinococcosis in humans

The immunodiagnosis of echinococcosis has been comprehensively reviewed (Zhang *et al.*, 2003a). Over the past decade, diagnosis of CE has improved due to the use of new or more optimal methods for purification of *Echinococcus* antigens from somatic materials, by the application of molecular tools for parasite identification and the synthesis of recombinant diagnostic antigens and immunogenic peptides. These approaches have not only improved the sensitivity and specificity of tests for diagnosis of CE but they have also allowed more reliable characterization of the biological status of parasite materials (Zhang and McManus, 2006; Siles-Lucas and Gottstein, 2001).

The long history of CE serodiagnosis has mainly involved the identification and characterization of specific *E. granulosus* antigens. The lipoproteins antigen B (AgB) and antigen 5 (Ag5), the major components of HCF, have been the two molecules that have received wide attention in regards to diagnosis. Both antigens have been well characterized in terms of their molecular features and diagnostic potential (Zhang *et al.*, 2003a). Although AgB and Ag5 have proved to be diagnostically valuable, there are difficulties related to their lack of sensitivity and specificity and problems with the standardization of their use. Cross-reactivity with antigens from other parasites, notably other taeniid cestodes, is a major problem. IgE cross-reaction with other parasites (Wattal *et al.*, 1988; Force *et al.*, 1992) is common.

Data in the literature for sensitivity and cross-reactivity of serum anti-*Echinococcus* IgE differ significantly; most studies report high specificity (99–100%) (Marinova *et al.*, 2011; Chamekh *et al.*, 1992; Khabiri *et al.*, 2006; Afferni *et al.*, 1984; Sjolander *et al.*, 1989; Zarzosa *et al.*, 1999)

A recent study using a large panel of sera showed that crude or purified antigens from parasite or hydatid cyst fluid generated a reasonable high specificity (Feng *et al.*, 2010). By using 857 sera from confirmed CE patients and 42 sera from AE patients and 697 sera with different infection and medical conditions showed an overall of 93.4% of specificity with relative low sensitivity 57.4–68.4% (Table 1) (Feng *et al.*, 2010).

Antigen	Test	Number of subjects tested			Sensitivity (%)	Specificity (%)	Reference
		CE	Healthy	Other diseases			
HBLF	LA	119	37	54	86	87.9	(Barbieri <i>et al.</i> , 1993)
HFF	IHA	204	90	53	54	100	(Ortona <i>et al.</i> , 2000)
HFF	IgE IB	204	90	53	80	96	(Ortona <i>et al.</i> , 2000)
HFF	IEP	204	90	53	31	100	(Ortona <i>et al.</i> , 2000)
HCF	IgG ELISA	71	45	62	64	80	(Verastegui <i>et al.</i> , 1992)
FBHCF	IgG ELISA	119	37	54	83	86.8	(Barbieri <i>et al.</i> , 1993)
sWHF	IgG ELISA	111/Li	0	0	89	nd	(Babba <i>et al.</i> , 1994)
sWHF	IgG ELISA	122/Lu	0	0	78	nd	(Babba <i>et al.</i> , 1994)
FBHCF	IgG ELISA	90	28	88	84	60	(Barbieri <i>et al.</i> , 1998)
HCF	IgG ELISA	87	200	339	94	82.3	(Poretti <i>et al.</i> , 1999)
HCF	IgG ELISA	42	15	41	81	95	(Irabuena <i>et al.</i> , 2000)
HFF	IgG ELISA	204	90	53	72	97	(Ortona <i>et al.</i> , 2000)
ppHCF	IgG ELISA	70	30	73	89	40.8	(Jiang <i>et al.</i> , 2001)
HCF	IgG ELISA	129	203	65	78	97	(Virginio <i>et al.</i> , 2003)
HCF	IgG ELISA	59	15	55	79	73	(Lorenzo <i>et al.</i> , 2005)
HCF	IgG ELISA	26	10	45	96	100	(Al-Sherbiny <i>et al.</i> , 2004)
HCF	IgG Dip	26	10	45	100	91	(Al-Sherbiny <i>et al.</i> , 2004)
HCF	IgG WB	71	45	62	65	91	(Verastegui <i>et al.</i> , 1992)
HCF AgB	IgG Dot-IB	875	5	739	68.4	93.4	(Feng <i>et al.</i> , 2010)
HCF AgB	IgG ELISA	857	5	739	57.4	93.4	(Feng <i>et al.</i> , 2010)
HCF AgB	IgE WB	324	70	500	86.4	92	(Li <i>et al.</i> , 2003b)
HCF	IgG WB	26	10	45	100	91	(Al-Sherbiny <i>et al.</i> , 2004)
HCF	IgE ImCAP	155	110	58	73.6	99.1	(Marinova <i>et al.</i> , 2011)
HCF	IgG ELISA	155	110	58	90.3	90.9	(Marinova <i>et al.</i> , 2011)
HCF	IgG WB	155	110	58	90.1	94.5	(Marinova <i>et al.</i> , 2011)

Table 1. Features of assays for immunodiagnosis of cystic echinococcosis based on hydatid cyst fluid antigens and native proteins from *E. granulosus* Abbreviations: HFF - hydatid fluid fraction, rich in Ag5 and AgB; HBLF - heparin-binding lipoprotein fraction; ppHCF - partially purified HCF; FBHCF - fertile bovine hydatid cyst fluid; Li - liver; Lu - lung; sWHF - sheep whole hydatid cyst fluid; IHA - indirect haemagglutination assay; LA - Latex agglutination assay; IEP - immunoelectrophoresis; ELISA - enzyme - linked immunosorbent assay; WB - Western blotting; Dip, dipstick; ImCAP, immunoCAP system.

4.2.1 *E. granulosus* antigen B

E. granulosus antigen B (AgB), a polymeric lipoprotein with a molecular weight of 120 kDa, is a highly immunogenic molecule, a characteristic that underpins its value in serodiagnosis (Table 2). AgB can be measured in patient blood as circulating antigen (Kanwar *et al.*, 1994; Kanwar and Kanwar, 1994; Liu *et al.*, 1993). It has a molecular size of circa 8 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Lightowlers *et al.*, 1989; Shepherd and McManus, 1987) under reduced conditions. The function of AgB in the parasite’s biology is not completely elucidated, but several studies have shown that the molecule may be involved in the modulation of the host immune response; for instance, as a protease inhibitor that inhibits neutrophil chemotaxis (Shepherd *et al.*, 1991; Virginio *et al.*, 2007), promoting a non-protective Th2 response by interfering with monocyte differentiation, and by modulating DC maturation (Kanan and Chain, 2006; Rigano *et al.*, 2007). One study has suggested that AgB could be involved in the processes of lipid uptake or detoxification (Chemale *et al.*, 2005).

AgB	Test	Number of subjects tested			Sensitivity (%)	Specificity (%)	Reference
		CE	Healthy	Other diseases			
Gel-EF	IgG ELISA	204	90	53	74	100	(Ortona <i>et al.</i> , 2000)
pp	IgG ELISA	90	28	86	77	85	(Gonzalez-Sapienza <i>et al.</i> , 2000)
pp	IgG ELISA	191	50	133	79	98	(Shambesh <i>et al.</i> , 1997)
pp	IgG ELISA	81	-	98	89	86	(Wen and Craig, 1994)
AEC	IgG ELISA	31	29	87	77	82	(Rott <i>et al.</i> , 2000)
mAb-AP	IgG ELISA	90	28	88	77	86	(Barbieri <i>et al.</i> , 1998)
pp	IgG1 ELISA	81	-	98	58	92	(Wen and Craig, 1994)
pp	IgG1 ELISA	191	50	133	57	100	(Shambesh <i>et al.</i> , 1997)
pp	IgG2 ELISA	81	-	98	53	94	(Wen and Craig, 1994)
pp	IgG3 ELISA	81	-	98	46	95	(Wen and Craig, 1994)
pp	IgG4 ELISA	210	47	79	63	81	(McVie <i>et al.</i> , 1997)
pp	IgG4 ELISA	191	50	133	38	99	(Shambesh <i>et al.</i> , 1997)
pp	IgG4 ELISA	81	-	98	73	91	(Wen and Craig, 1994)
pp	IgG4 ELISA	210	47	79	63	81	(McVie <i>et al.</i> , 1997)
AgB	IgG ELISA	129	203	65	60	93	(Virginio <i>et al.</i> , 2003)
AgB	IgG ELISA	36	36	-	91.7	97.2	(Kalantari <i>et al.</i> , 2010)
AgB	IgG ELISA	59	15	55	80	77	(Lorenzo <i>et al.</i> , 2005)
AgB	IgG ELISA	40	70	40	92	97	(Sadjjadi <i>et al.</i> , 2007)
pp	IgG dELISA	210	47	79	93	65	(McVie <i>et al.</i> , 1997)
18kDa	IgG WB	69	82	63	10	77	(Jiang <i>et al.</i> , 2001)
8 kDa	IgG WB	35	200	339	71	97	(Poretti <i>et al.</i> , 1999)
8 kDa	IgG WB	52p	200	339	60	97	(Poretti <i>et al.</i> , 1999)
8-34kDa	IgG WB	173	29	66(AE)	85	65	(Ito <i>et al.</i> , 1999)
8-34kDa	IgG WB	35	200	339	91	94	(Poretti <i>et al.</i> , 1999)
8-34kDa	IgG WB	52p	200	339	81	94	(Poretti <i>et al.</i> , 1999)
Gel-EF	IgG WB	204	90	53	66	100	(Ortona <i>et al.</i> , 2000)
pp	IgG WB	158	29	152	92	69	(Ito <i>et al.</i> , 1999)
pp	IgG WB	173	29	115	92	100	(Ito <i>et al.</i> , 1999)
8kDa	IgG WB	44	-	43	47.7	51.2	(de la Rue <i>et al.</i> , 2010)
16kDa	IgG WB	44	-	43	45.5	67.4	(de la Rue <i>et al.</i> , 2010)
24kDa	IgG WB	44	-	43	68.2	62.8	(de la Rue <i>et al.</i> , 2010)

Table 2. Features of assays for immunodiagnosis of cystic echinococcosis using native antigen B Abbreviations: Gel-EF - eluted fractions from SDS-PAGE; pp - partial purification; AEC - anion exchange chromatography; mAb - AP - affinity purification by monoclonal antibody; 52p - days post-surgery; AE - alveolar echinococcosis; ELISA - enzyme - linked immunosorbent assay; dELISA - dot - ELISA; WB - Western blot.

AgB is a gene family containing at least 10 genes in 5 subfamilies (Shepherd *et al.*, 1991; Fernandez *et al.*, 1996; Chemale *et al.*, 2001; Arend *et al.*, 2004; Mamuti *et al.*, 2007; Zhang *et*

al., 2010), which are differentially expressed in different stages of *E. granulosus* (Mamuti *et al.*, 2007; Zhang *et al.*, 2010). Several AgB cDNAs, such as rAgB8/1 and rAgB8/2, have been cloned, expressed as recombinant proteins and used for diagnosis. AgB8/2 provided the highest diagnostic sensitivity (84-93.1%) and specificity (98-99.5%) (Rott *et al.*, 2000; Virginio *et al.*, 2003). Furthermore, the IgG4 response against AgB appears to be the subclass of choice for serological testing (Siracusano *et al.*, 2004).

The sensitivity and specificity of native *E. granulosus* cyst fluid antigens and native and recombinant AgB are shown in Tables 1-3. These studies used small panel of sera and

Antigen	Assay method	Number of subjects tested			Sensitivity (%)	Specificity (%)	Reference
		CE	Healthy	Other diseases			
rAgB.MBP	IgG4 ELISA	210	47	79	65	91	(McVie <i>et al.</i> , 1997)
EG55-GST	IgG ELISA	64	39	105	89	72	(Helbig <i>et al.</i> , 1993)
rAgB8/1	IgG ELISA	31	29	87	55	80	(Rott <i>et al.</i> , 2000)
rAgB8/2	IgG ELISA	31	29	87	84	98	(Rott <i>et al.</i> , 2000)
rEgPS-3-GST	IgG ELISA	119	44	123	74	87	(Leggatt and McManus, 1994)
P176	IgG ELISA	90	28	86	80	93	(Gonzalez-Sapienza <i>et al.</i> , 2000)
P175	IgG ELISA	90	28	86	49	94	(Gonzalez-Sapienza <i>et al.</i> , 2000)
P177	IgG ELISA	90	28	86	38	92	(Gonzalez-Sapienza <i>et al.</i> , 2000)
P65	IgG ELISA	90	28	86	44	96	(Gonzalez-Sapienza <i>et al.</i> , 2000)
pGu4	IgG ELISA	90	28	86	18	98	(Gonzalez-Sapienza <i>et al.</i> , 2000)
pGU4	IgG ELISA	31	29	87	26	-	(Rott <i>et al.</i> , 2000)
p65#	IgG ELISA	90	28	88	34-48	80-97	(Barbieri <i>et al.</i> , 1998)
pGU4#	IgG ELISA	90	28	88	12-18	96-100	(Barbieri <i>et al.</i> , 1998)
rAgB.MBP	IgG dELISA	210	47	79	74	88	(McVie <i>et al.</i> , 1997)
p65	IgG dELISA	25	9	8	64	100	(Leggatt and McManus, 1994)
rAgB-GST	IgG WB	204	90	53	72	100	(Ortona <i>et al.</i> , 2000)
P176	IgG ELISA	59	15	55	63	82	(Lorenzo <i>et al.</i> , 2005)
rAgB1	IgG ELISA	59	15	55	68	88	
rAgB1		102	95	68	88.2	80.9	
rAgB2	IgG ELISA	102	95	68	91.2	93	(Hernandez-Gonzalez <i>et al.</i> , 2008)
rAgB-GST	IgG IB	120	24	97	79.2	81	(Lv <i>et al.</i> , 2009)
rAgB	IgG ELISA	246		173	77.6		(Li <i>et al.</i> , 2010)

Table 3. Features of assays for immunodiagnosis of cystic echinococcosis based on using recombinant antigen B and antigen B peptides Abbreviations: ELISA - enzyme-linked immunosorbent assay; dELISA - dot enzyme - linked immunosorbent assay; ELISAs - sandwich ELISA; rEgPS - recombinant protoscolex protein; GST- glutathione S-transferase; rAgB - recombinant antigen B; MBP - maltose binding protein; # - coated with different buffer; WB-Western blot

showed generally both high sensitivity and specificity (Tawfeek *et al.*, 2011; Kalantari *et al.*, 2010; Abdi *et al.*, 2010; Lv *et al.*, 2009; Sadjjadi *et al.*, 2007; Virginio *et al.*, 2003). Cross-reactions, mainly with sera from AE patients, have been reported to be up to 47.7% (de la Rue *et al.*, 2010).

In addition, a number of AgB peptides have been synthesized and used in ELISA for diagnostic purposes (Table 3). Peptide antigens have been considered as a way to enhance specificity and efforts have been made to define discrete epitopes of AgB and other molecules that could be mimicked by synthetic peptides. However, a double-blind, randomized multicenter comparison of the diagnostic performance of six major antigens (namely, HCF, native AgB, two recombinant AgB subunits, an AgB-derived synthetic peptide, and recombinant cytosolic malate dehydrogenase) against the same serum collection showed the AgB-derived synthetic peptide provided relatively poor specificity, with instead, one of the recombinant AgB subunits (AgB8/1) being recommended as the standard antigen for laboratory analysis (Lorenzo *et al.*, 2005).

4.2.2 *E. granulosus* antigen 5

E. granulosus antigen 5 (Ag5) is a very high molecular weight (approximately 400 kDa) lipoprotein complex composed of 57- and 67-kDa components that, under reducing conditions, dissociate into 38- and 22- to 24-kDa subunits in SDS-PAGE. Historically, one of the most used immunodiagnostic procedures for CE was the demonstration of serum antibodies precipitating antigen 5 (arc 5) by immunoelectrophoresis or similar techniques. Table 4 shows the performance of antigen 5 for serological diagnosis of human CE. Loss of sugar determinants on antigen 5 can reduce the antigenicity of the native protein (Lorenzo *et al.*, 2005a). The potential value of specific antibodies of different IgG subclasses and IgE in serological diagnosis of CE using an ELISA based on Ag5 has been evaluated (Khabiri *et al.*, 2006). The presence of IgG1 was demonstrated in all sera from 58 patients with CE. The most discriminatory and specific antibodies found in this study were IgG4 and IgE. Only one false-positive reaction was observed with IgG4 and no IgE cross-reactivity occurred with 40 sera from healthy controls. In 36 sera from patients with parasitic diseases other than CE, two false-positive reactions with IgG4 were observed but none occurred with IgE. In immunoblotting, it was shown that the IgG1 subclass was responsible for cross-reactivity of human antibodies that reacted with the 38kDa subunit of Ag5. IgG4 and IgE antibodies could not recognize the 38kDa subunit and under non-reducing conditions reacted with the 57kDa subunit without any cross-reactivity with other parasites. These results demonstrated that IgG4 and IgE are the most important antibodies for serological diagnosis of CE in an Ag5-based immunoassay system. Like AgB, Ag5 has been cloned, expressed and tested for diagnostic performance but the recombinant protein showed low sensitivity (Table 4).

4.2.3 Other recombinant proteins and approaches for immunodiagnosis

Table 4 lists the diagnostic performance of some other recombinant *E. granulosus* proteins cloned recently. Li *et al.* (2003b) cloned a fragment designated as EpC1 and tested 896 human serum samples including 324 sera samples from patients with CE, 172 from patients with neurocysticercosis, 89 from patients with AE, and 241 from patients with other infections or clinical presentations, as well as 70 from confirmed-negative control subjects.

Antigen	Test	Number of objects tested			Sensitivity (%)	Specificity (%)	Reference
		CE	Heathy	Other disease			
Arc5	IEP	35	200	289	63	97.2	(Poretti et al., 1999)
Arc5	IEP	52p*	200	289	58	97.2	(Poretti et al., 1999)
mAb-AP	IgG ELISA	90	28	88	50	92	(Barbieri et al., 1998)
mAb-AP	IgG ELISA	39	29	51	54	89	(Gonzalez et al., 2000)
P89-122#	IgG ELISA	90	28	88	14-21	77-100	(Barbieri et al., 1998)
rP-29	IgG ELISA	39	29	51	61	80	(Gonzalez et al., 2000)
P89-122#	IgG ELISA	39	29	51	44	100	(Gonzalez et al., 2000)
pAg 5	IgG ELISA	111(Li)	nd	nd	89	nd	(Babba et al., 1994)
pAg 5	IgG ELISA	122(Lu)	nd	nd	78	nd	(Babba et al., 1994)
nAg5	IgG ELISA	58	40	36	100	70.2	(Khabiri et al., 2006)
nAg5	IgG1 ELISA	58	40	36	100	70.2	(Khabiri et al., 2006)
nAg5	IgG4 ELISA	58	40	36	75.8	93	(Khabiri et al., 2006)
nAg5	IgE ELISA	58	40	36	70	100	(Khabiri et al., 2006)
rAg5	IgG ELISA	34	18	36	65	89	(Lorenzo et al., 2005b)
rAg5-38	IgG ELISA	34	18	36	21	97	(Lorenzo et al., 2005b)
EpC1	IgG WB	324	70	500	88.7	95.6	(Li et al., 2003b)
rEgcMDH	IgG ELISA	59	15	55	45	83	(Lorenzo et al., 2005a)
HSP20	IgG1/4 WB	95	37	-	64	-	(Vacirca et al., 2011)
Eg19	IgG WB	97	37	58	10	100	(Delunardo et al., 2010)
E14t	IgG ELISA	102	95	68	35.3	91.7	(Hernandez-Gonzalez et al., 2008)
C317	IgG ELISA	102	95	68	58.8	80.9	(Hernandez-Gonzalez et al., 2008)
P5	IgG WB	60	-	-	97	-	(Zhang et al., 2007)

* post-treatment. Abbreviations: Li - liver; Lu - lung; mAb-AP - affinity purified using monoclonal antibody; # - coated with different buffer; pAg5 - purified antigen 5; nd - not determined.

Table 4. Features of assays for immunodiagnosis of cystic echinococcosis based on using native, recombinant and synthetic peptides of antigen 5 and other proteins

The fusion protein yielded an overall sensitivity of 92.2% and an overall specificity of 95.6%. The combined levels of sensitivity and specificity achieved with the rEpC1-GST fusion protein for diagnosis of CE were exceptional, taking into account the large panel of serum samples that were tested (Li et al., 2003b). The cDNA sequence coding for EpC1 has high amino acid sequence identity to a paralogue from *Taenia solium*, the cause of neurocysticercosis (NCC). To determine diagnostic antibody-binding regions on EpC1 recognized specifically by CE sera, 10 truncated regions (P1-10) of the immunogenic protein were expressed in *Escherichia coli* and subjected to immunoblotting (Zhang et al., 2007). One peptide, designated peptide 5 (P5, fused with glutathione-S-transferase (GST)) was positively recognized by sera from mice experimentally infected with oncospheres of *E. granulosus* and sera from surgically confirmed CE patients. Sera from NCC patients did not react with any of the peptides used. There are four amino acid substitutions in P5 compared with the *T. solium* sequence and these may form part of the epitope inducing CE-specific antibody. Ninety-seven per cent (58 of 60) of sera from confirmed CE patients recognized P5-GST.

By using ELISA, a cytosolic isoform of malate dehydrogenase (EgcMDH), an EF-hand calcium-binding protein (EgCaBP2), and a full-length (EgAFFPf) and a truncated form

(EgAFFPt, aa 261–370) of actin yielded sensitivities between 58.6% and 89.7%, and three of them were considered of complementary value (Virginio *et al.*, 2003).

A purified alkaline phosphatase (EgAP) extracted from *E. granulosus* hydatid cyst membranes has shown exceptional diagnostic characteristics with 100% specificity without any decrease in sensitivity (100%) with significant potential for use in routine diagnosis and follow-up of CE patients (Mahmoud and Abou Gamra, 2004). This mirrors the diagnostic value previously shown for purified alkaline phosphatase (pAP) from *E. multilocularis* metacestodes (Zhang *et al.*, 2003a).

Purified recombinant thioredoxin peroxidase from *E. granulosus* (TPxEg) was used to screen sera from heavily infected mice and patients with confirmed hydatid infection (Li *et al.*, 2004). Only a portion of the sera reacted positively with the EgTPx-GST fusion protein in Western blots (69.3% specificity and 39% sensitivity with human sera), suggesting that EgTPx may form antibody-antigen complexes or that responses to the EgTPx antigen may be immunologically regulated.

IB revealed anti-HSP20 antibodies in a higher percentage of sera from patients with active disease than in sera from patients with inactive disease (Vacirca *et al.*, 2011). A comparison of the ImmunoCAP system for testing serum IgE compared with IgG-ELISA and IgG-Western blotting revealed the former test had a higher specificity and lower cross-reactivity with a sensitivity of 73.6% (Marinova *et al.*, 2011).

4.3 Immunodiagnosis of alveolar echinococcosis in humans

A number of advances in the serodiagnosis of AE have occurred in the past 10 years or so. These include identification of novel antigens, such as Em2 and Em18. Em2 was purified originally from *E. multilocularis* metacestode tissue extracts by affinity chromatography (Gottstein, 1985). Using AE sera to screen a cDNA library, a clone termed II/3 was cloned and showed very high performance for diagnosis (Vogel *et al.*, 1988). A commercial kit based on Em2 and recombinant truncated protein II/3 so called Em2^{Plus} ELISA has shown high performance of AE diagnosis (Gottstein *et al.*, 1993).

Em18, an 18-kD antigen from *E. multilocularis*, is highly specific for detection of AE (Ito *et al.*, 2003a; Xiao *et al.*, 2003). An ELISA system using the recombinant Em18 antigen (RecEm18) could differentially distinguish AE from CE (Ito *et al.*, 2003a) and may be used for monitoring surgical and/or chemotherapeutic treatment and the follow up of AE patients after treatment (Fujimoto *et al.*, 2005). In order to compare the sequential responses of IgG subclasses to Em18 in sera from patients with AE, a total of 225 sera from 36 patients at different clinical stages according to the WHO-PNM staging system (Pawlowski *et al.*, 2001) were tested. The levels of serum IgG and IgG4 against Em18 correlated with the PNM stages compared with sera from patients receiving no treatment (Tappe *et al.*, 2010).

Antibody-screening was performed by (Reiter-Owona *et al.*, 2009) using ELISA, IHA and IFAT, and confirmatory testing was done using the commercialized *E. multilocularis*-specific Em2plus-ELISA versus an in-house *E. multilocularis*-specific Em10-ELISA. The study showed that the Em2plus-ELISA reacted with 23.5% CE-positive sera, whereas the Em10-ELISA did not exhibit any cross-reactivity. In sera from patients with AE, confirmation by

both ELISAs was achieved in 57.6% of cases, mostly in patients with an advanced stage of the disease and high antibody titers in the screening assays. False-negative reactions with both ELISAs occurred in 30.3% cases, mostly in patients who had low antibody levels in the screening tests. The Em2plus-ELISA exhibited fewer false-negative reactions than the Em10-ELISA. WB confirmed the positive results of both assays and was the assay with the highest reliability with different stages of CE and AE, followed by the Em2plus-ELISA for AE. High antibody titres in the screening assays favour the detection of species-specific antibodies to either CE or AE (Reiter-Owona *et al.*, 2009). The features of assays currently available for immunodiagnosis of alveolar echinococcosis using different antigens are presented in Table 5.

Antigen	TEST	Number of subjects tested			Sensitivity (%)	Specificity (%)	Reference
		AE	Healthy	Other disease			
Em2 ^{plus}	IgG ELISA	140	500	400	97.1	90.2	(Gottstein <i>et al.</i> , 1993)
Em2	IgG ELISA	140	500	400	89.3	98.0	(Gottstein <i>et al.</i> , 1993)
Em2 ^{plus}	IgG ELISA	47	-	-	81	-	(Bart <i>et al.</i> , 2007)
Em2	IgG ELISA	62	-	97	90	65	(Muller <i>et al.</i> , 2007)
rEm10	IgG ELISA	74	39	95	93.2	96.5	(Helbig <i>et al.</i> , 1993)
II/3-10	IgG ELISA	140	500	400	86.4	96.8	(Gottstein <i>et al.</i> , 1993)
CH-10	IgG ELISA	140	500	400	96	39-97	(Gottstein <i>et al.</i> , 1993)
N3C	IgG ELISA	140	500	400	96	18-94	(Gottstein <i>et al.</i> , 1993)
pAP	IgG ELISA	37	37	95	100	100	(Ravinder <i>et al.</i> , 1997)
Em70	IgG ELISA	39	32	115	100	99	(Korkmaz <i>et al.</i> , 2004)
Em90	IgG ELISA	39	32	115	100	99	(Korkmaz <i>et al.</i> , 2004)
II/3-10	IgG ELISA	62	-	97	79	92	(Muller <i>et al.</i> , 2007)
Em18	IgG ELISA	44	30	99	91	89.1	(Jiang <i>et al.</i> , 2001)
rEm13	IgG ELISA	28	-	72	82.1	100	(Frosch <i>et al.</i> , 1993)
rEm18	IgG IB	66	29	259	97	96.9	(Ito <i>et al.</i> , 1999)
rEm18	IgG IB	33	82	99	91	92.3	(Jiang <i>et al.</i> , 2001)
rEm18	ELISA	19	0	189	100	99	(Xiao <i>et al.</i> , 2003)
EmII	Dot-IB	42	-	-	91.1	-	(Feng <i>et al.</i> , 2010)

Table 5. Features of assays for immunodiagnosis of alveolar echinococcosis using different antigens

Although AE serological diagnosis has made great strides to practical application, a recent expert consensus for alveolar echinococcosis diagnosis (Brunetti *et al.*, 2010) stated that the most patients with AE are diagnosed at a later stage, which significantly impacts on quality of treatment. There is an urgent need for the early diagnosis. The application of new techniques is a way to address these issues; for example, recent microarray analysis identified 5 genes (Gapdh, Est1, Rlp3, Mdh-1, Rpl37) exhibiting a high level of congruency, and these may provide new diagnostic targets to predict disease status and progression (Gottstein *et al.*, 2010).

4.4 Circulating antigen detection

Antibody detection is likely to indicate exposure to an *Echinococcus* infection, but it may not necessarily point to the presence of an established, viable infection, or the disease. Serum

antibodies may persist for a prolonged period, reaching up to 10 years after hydatid cyst removal (Li *et al.*, 2004). In addition, the degree of antibody response may be related to the location and condition of a mature hydatid cyst. For instance, hydatid cysts in human lung, spleen, or kidney tend to be associated with lower serum antibody levels (Zhang and McManus, 2006). Furthermore, in *Echinococcus*-endemic villages, up to 26% or more of the general population may have antibodies to HCF antigens, but with only about 2% of the villagers having hydatid cysts (Craig *et al.*, 1986; Chai *et al.*, 1989; Gavidia *et al.*, 2008), indicating that the antibody levels may not necessarily reflect the true prevalence of CE.

Antigen detection assays depend principally on the binding of specific polyclonal or monoclonal antibodies to parasite antigen present in serum or urine. A number of different assays have been developed to detect echinococcal antigens. The standard double antibody sandwich ELISA is a common method for measuring the presence and/or concentration of circulating parasite antigens. In the test, antibody raised to the targeted protein is coated onto a microtiter plate to capture antigen (Fig 3). The same antibody, that is enzyme labeled, is commonly used in the tertiary layer of the assay. This type of antigen capture therefore relies on the presence of multiple binding sites on the target antigens(s). Efforts to detect CAg in CE patients have been reviewed extensively by Craig (Craig *et al.*, 1986).

CAg in serum is normally in the form of a circulating immune complex (CIC) with some in free form. Therefore, the serum needs to be treated with acid buffer or polyethylene glycol (PEG) to release and concentrate the circulating antigens. Acidic treatment (0.2 M glycine/HCl) of CE patient serum is quite straightforward to dissociate CIC (Craig *et al.*, 1986). In a comparison of acid-treatment and PEG precipitation methods, all the sera of 30 confirmed positive cases of CE had detectable levels of antigen in the acid-treated sera (Craig, 1993). However, 23 (77%) and 26 (87%) sera of 30 confirmed cases had free antigen as well as CIC of an 8 kDa antigen in the untreated and in the PEG precipitated sera, respectively. None of the sera from other patients with parasitic infections or viral hepatitis had any detectable levels of 8 kDa antigen in the untreated, acid-treated or PEG-precipitated serum samples. These investigations, therefore, suggested that the demonstration of circulating antigen employing monospecific antibodies to affinity purified 8 kDa antigen in acid-treated sera is more efficient than the detection of free circulating antigen or CIC in untreated or in PEG-precipitated sera (Kanwar *et al.*, 1994).

IgM CICs tend to be positively associated with active hydatid disease (Craig *et al.*, 1986; Matossian *et al.*, 1992). Combining measurement of circulating antibody, CICs and CAg resulted in an increase from 77% to 90% compared to measurement of serum antibody alone (Moosa and Abdel-Hafez, 1994). Antigens in soluble CICs from CE patients have been characterized by separating them on SDS-PAGE (Craig *et al.*, 1986) or by ion-exchange fast protein liquid chromatography (FPLC) (Bonifacino *et al.*, 1993). Both studies indicated a candidate antigen detectable in serum with an approximate relative molecular mass of 60-67 KDa, and which is also present in cyst fluid.

Comparison of CAg and IgG antibody using ELISA, together with Western blotting, showed a relatively low sensitivity (43%) for detection of specific serum antigen in CE, compared to 75% for IgG antibodies (Craig, 1997). However, the specificity of this CAg ELISA was 90% when tested against sera from AE patients and 100% against human cysticercosis sera. The limited

cross-reactivity may be a way for practical diagnosis of CE in areas where AE and cysticercosis are co-endemic. The advantage of CAg detection is its high sensitivity for detecting CE in 54-57% of patients who are serum antibody negative (Moosa and Abdel-Hafez, 1994; Craig, 1997). CAg detection does appear, therefore, to be potentially useful as a secondary test for some suspected CE cases where antibody titers are low (Craig *et al.*, 1986; Schantz, 1988).

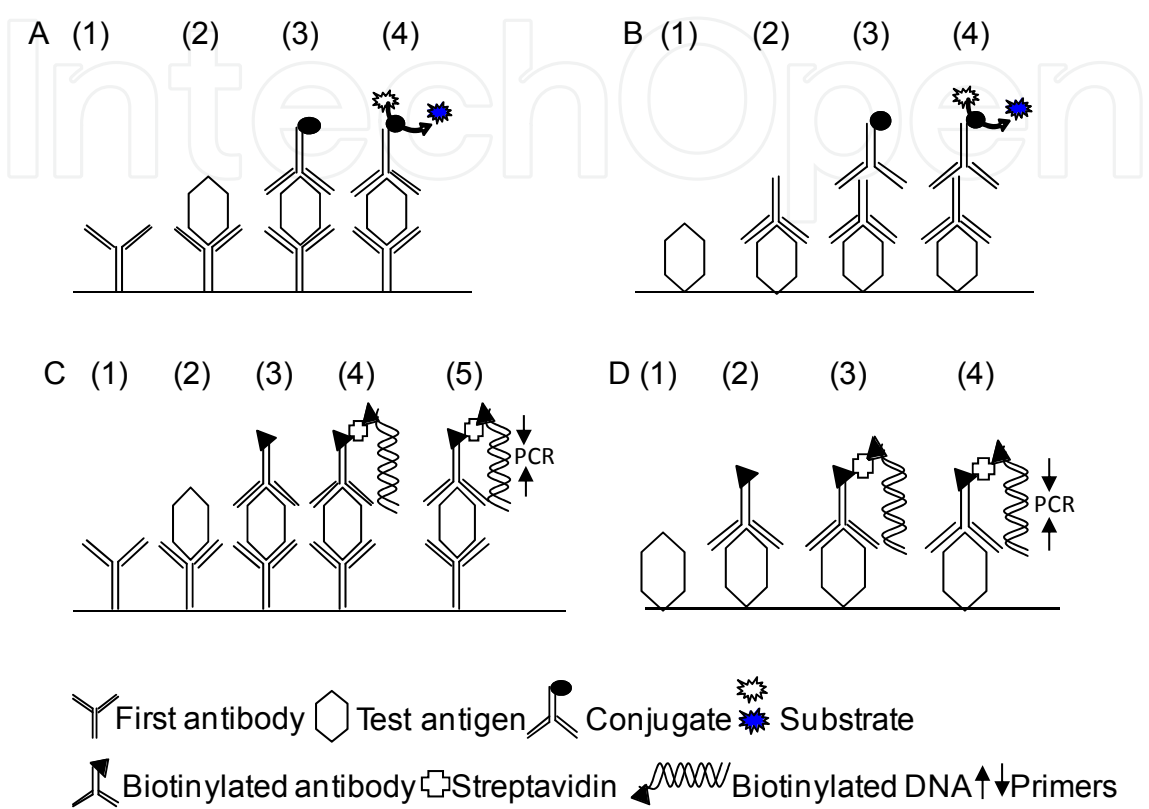


Fig. 3. Schematic of ELISA and immuno-PCR for detecting circulating antigen in serum. A. Sandwich ELISA. (1) Plate is coated with a capture antibody; (2) Serum sample is added, and any antigen present in the serum binds to the capture antibody; (3) Detecting antibody conjugate is added and binds to the antigen; (4) Substrate is added, and is converted by the enzyme to a detectable form. B. Direct ELISA. Plate is coated with diluted serum containing antigen; (2) Detecting antibody is added, and binds to antigen; (3) Enzyme-linked secondary antibody is added, and binds to detecting antibody; (4) Substrate is added, and is converted by the enzyme to a detectable form. C. Capture immuno-PCR. (1) Plate is coated with capture antibody; (2) Serum sample is added; (3) Biotinylated detecting antibody is added and binds to antigen; (4) Streptavidin and biotinylated reporter DNA are added, and the biotinylated antibody and biotinylated reporter DNA are linked by streptavidin; (5) Primers and PCR components are added and PCR or real- time PCR undertaken to quantify antigen. D, Non-capture immuno-PCR. Serum sample is coated on the plate and the remainder of the steps are as for the capture-immuno-PCR (C).

A combination of CAg and antibody detection has been shown to increase the sensitivity from 85% (antibody only) to 89% (antibody+CAg) in ELISA of 115 surgically confirmed hydatid patients, 41 individuals exhibiting other parasitic and unrelated diseases, and 69 healthy subjects(Barbieri *et al.*, 1994).

Urinary hydatid antigen detection by co-agglutination (Co-A) potentially represents a cost-effective and rapid test for diagnosis of CE in a rural or field setting. However, the lower sensitivity of Co-A for detection of antigen in the urine of a patient whose serum was positive for the antigen is possibly due to low levels of antigen in the urine (Ravinder *et al.*, 2000).

Although there has been no application to date for echinococcal diagnosis, a technique for antigen detection, called immuno-polymerase chain reaction (immuno-PCR) (Fig 3 C and D), has been developed (Sano *et al.*, 1992). It combines the molecular recognition of antibodies with the high DNA amplification capability of PCR. The procedure is similar to conventional ELISA but is far more sensitive, and, in principle, could be applied for the detection of single antigen molecules. Instead of an enzyme, a DNA molecule is linked to the detection antibody and serves as a template for PCR (Fig 3). The DNA molecule is amplified and the PCR product is measured by gel electrophoresis. An improvement of this method is to amplify the DNA fragment by real-time PCR, thereby eliminating post-PCR analysis. Furthermore, real-time PCR is extremely accurate and sensitive, which should make it possible to quantitate very low amounts of DNA-coupled detection antibody with high accuracy.

5. Immunodiagnosis of cystic echinococcosis in intermediate host animals

Research towards developing serological tests for the diagnosis of larval cestode infection in animals has been largely unsuccessful. Substantial problems remain, due to the frequent existence of multiple infections with different taeniid species, antigenic cross-reactivity between these related parasites, and the low level of specific antibody response to infection. Problems with poor specificity and sensitivity of traditional serological tests for cysticercosis and hydatidosis have prevented the development of any practical test for ante-mortem diagnosis of infection. An approach to the diagnosis of *Taenia* infection by detecting circulating parasite antigen (Onyango-Abuje *et al.*, 1996; Lightowlers, 1990; Harrison *et al.*, 1989) offers some prospect for the development of a practical diagnostic test for cysticercosis.

In comparison with the extensive investigations in humans, relatively little research has been directed toward the development of immunodiagnostic techniques for *E. granulosus* infection in domesticated animals such as sheep and cattle. Currently, diagnosis of CE in intermediate hosts is based mainly on necropsy procedures. However, up to 37% of animals classified as positive at necropsy may be actually false positives caused by unspecific granulomas, pseudo-tuberculosis, fatty degeneration, abscesses, caseous lymphadenitis, and larval stage of *Taenia hydatigena*, whereas false negative diagnoses may be due to small intra-parenchyma cysts (Larrieu *et al.*, 2001; Gatti *et al.*, 2007).

Accurate serological diagnosis of CE infection in livestock is necessary, but, as indicated above, difficult due to serological cross-reactions with several other species of taeniid cestodes including *Taenia hydatigena* and *T. ovis* (Lightowlers and Gottstein, 1995; Yong *et al.*, 1984). Furthermore, natural intermediate host animals produce very poor antibody responses to infection compared with the relatively high levels of specific antibody seen in human infection (Lightowlers and Gottstein, 1995). In sheep, (Lightowlers *et al.*, 1984) detected low levels of antibodies to AgB in the sera of some animals, whereas, others have

reported reasonable high antibody responses to this molecule (Kanwar and Kanwar, 1994; Ibrahim *et al.*, 1996). ELISA techniques using a variety of antigens have been applied to the immunodiagnosis of animal CE (Yong *et al.*, 1984). In experimentally infected sheep, antibodies to hydatid antigens can be detected as early as 4-6 weeks post-infection (Yong *et al.*, 1984). However, as referred to above, serological cross-reactions between *E. granulosus* and other cestodes limit the specific diagnosis of hydatid infection by ELISA using crude parasite antigens (Yong *et al.*, 1984). Affinity purification of crude antigens with antibodies from animals immunized with homologous antigen (Craig and Rickard, 1982), or affinity depletion of cross-reactive antigens with monoclonal antibody (Craig *et al.*, 1980), only partially reduces the cross-reactivity. Polysaccharide antigens from either the secretions produced during *in vitro* cultivation of *E. granulosus* PSC or from mouse hydatid cyst membranes by phenol extraction have been used to test sera from sheep (Ris *et al.*, 1987). Although the antibody responses were significantly higher than those of sheep infected with *T. hydatigena* or *T. ovis*, very high cross-reacting antibody responses in the sera from *T. hydatigena*-infected animals were detected with the antigenic secretions from PSC. Neither antigen was sufficiently sensitive or specific for routine serodiagnostic use (Ris *et al.*, 1987).

To develop an immunological method for the identification of sheep infected with *E. granulosus*, (Kittelberger *et al.*, 2002) used an ELISA with antigen comprising either a purified 8 kDa hydatid cyst fluid protein (8kDaELISA), a recombinant EG95 oncosphere protein (OncELISA) or a crude protoscolex preparation (ProtELISA). Sera used for the assay validations were obtained from 249 sheep infected either naturally or experimentally with *E. granulosus* and from 1012 non-infected sheep. The highest diagnostic sensitivity was obtained using the ProtELISA at 62.7 and 51.4%, depending on the cut-off. Assay sensitivities were lower for the 8kDaELISA and the OncELISA. Diagnostic specificities were high, ranging from 95.8 to 99.5%, depending on the ELISA type and cut-off level chosen. A few sera from 39 sheep infected with *T. hydatigena* and from 19 sheep infected with *T. ovis* were recorded as positive. Western immunoblot analysis revealed that the dominant antigenic components in the crude protoscolex antigen preparation were macromolecules of about 70-150 kDa, most likely representing polysaccharides. This study demonstrated that the ProtELISA was the most effective immunological method of those assessed for detection of infection with *E. granulosus* in sheep. Because of its limited diagnostic sensitivity of about 50-60%, it could be useful for the detection of the presence of infected sheep on a flock basis but cannot be used for reliable identification of individual animals infected with *E. granulosus*.

In a later study, Simsek and Koroglu (2004) investigated the antigenic characteristics of hydatid cyst fluid in sheep by SDS-PAGE to evaluate the sensitivity and specificity of HCF-ELISA and immunoblotting for diagnosis of sheep hydatidosis. One band with a molecular weight of 116 kDa showed 88% sensitivity and 84% specificity in the immunoblot assay. Sensitivity (60%) was less but specificity was higher (94%) with the HCF-ELISA (Simsek and Koroglu, 2004). Ghorbanpoor *et al.* (2006) were able to detect specific circulating antigens or antibodies in the serum and urine of 13 experimentally infected sheep.

6. DNA techniques

In addition to imaging and serological tests, identification of *Echinococcus* infection via PCR-based assays and DNA sequencing using tissue biopsy from the patients can be of choice for

confirming diagnosis of echinococcosis for these unconfirmed and complicated cases. DNA techniques are now available that allow the unambiguous identification of *Echinococcus* species and *E. granulosus* strains using metacestode material excised from intermediate and human hosts provide a major new approach to the diagnosis of echinococcosis (Yang *et al.*, 2006; McManus, 2006; McManus and Thompson, 2003; Thompson and McManus, 2001). Detection or amplification of *E. multilocularis* nucleic acids in clinical samples has been used in diagnosis of AE infections in patients (Myjak *et al.*, 2003).

7. The challenges and future directions

Almost all available immunodiagnostic techniques, including methods for detecting specific antibodies and circulating parasite antigens in serum or other body fluids, have been applied for diagnosing echinococcosis. However, all the tools developed to date, are generally applicable for laboratory research purposes only. None of the available diagnostic tools, kits or methods are generally accepted by clinical physicians. Nevertheless, such serological tools are potentially important for epidemiological studies, confirmation of infection status and the treatment and the monitoring of control programs and efforts should continue so that new assays for improved, practical diagnosis of echinococcosis are developed.

8. Conclusion

This Chapter provides an update of recent progress in research on the immunology and serological diagnosis of echinococcosis caused by *E. granulosus* and *E. multilocularis*. Cystic echinococcosis (CE) is characterized by long term growth of larval cysts in humans and other intermediate hosts, whereas, alveolar echinococcosis is defined by its chronic progression in human liver, resulting in high mortality. Although the host-parasite interplay, in most cases of AE and CE, appears to be harmonious and clinically asymptomatic for a long period after infection, the host does produce detectable humoral and cellular responses against the causative parasites. Antibody responses against early *E. granulosus* infection are weak being, usually, undetectable in the early two to three weeks following infection. During the establishment stage, the parasite produces significant quantities of antigens that modulate the immune response including antibody production, which is essential for serodiagnostic measurement.

It is clear that the improvement of immunodiagnostic methods for echinococcosis has greatly contributed to a better understanding of the prevalence and the epidemiology of the infection. Immunodiagnostic tests will also provide a valuable tool in measuring the impact of the disease on human health and on animal production, data that are still missing in most endemic areas. The assays may contribute to the diagnosis of CE and AE and the follow-up of treatment. Ultrasound and X-ray imaging methods are generally inaccessible and/or too expensive for the rural population at risk. Under these conditions, serology may provide the only tool for diagnosis of the infection. Finally, efforts should continue to provide cheap, reliable and standardised serodiagnostic methods more widely available for both AE and CE.

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