MINIREVIEW

Recent advances in the immunology and diagnosis of echinococcosis

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Abstract

Echinococcosis is a cosmopolitan zoonosis caused by adult or larval stages of cestodes belonging to the genus *Echinococcus* (family Taeniidae). The two major species of medical and public health importance are *Echinococcus granulosus* and *Echinococcus multilocularis*, which cause cystic echinococcosis and alveolar echinococcosis, respectively. Both cystic echinococcosis and alveolar echinococcosis are serious diseases, the latter especially so, with a high fatality rate and poor prognosis if managed inappropriately. This review highlights recent advances in immunity to infection and vaccination against both parasites in their intermediate and definitive hosts and procedures for diagnosis of cystic echinococcosis and alveolar echinococcosis, including the value of immunodiagnostic and DNA approaches. There is discussion also of progress in genomics and related technologies that is providing valuable insights on the functional biology of the *Echinococcus* organisms. These studies will underpin future research that will reveal a better understanding of the *Echinococcus*-host interplay, and suggest new avenues for the identification of additional targets for diagnosis, vaccination and chemotherapy.

Introduction

Echinococcosis is a cosmopolitan parasitic zoonosis caused by adult or larval stages of cestodes belonging to the genus *Echinococcus* (family Taeniidae). Larval infection (hydatid disease; hydatidosis) is characterized by long-term growth of metacestode (hydatid) cysts in the intermediate host. The two major species of medical and public health importance are *Echinococcus granulosus* and *Echinococcus multilocularis*, which cause cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively. *E. granulosus* has a cosmopolitan distribution (McManus *et al*., 2003) and *E. multilocularis*, which is distributed in the northern hemisphere, is recognized as an emerging zoonosis in some regions of Europe and Eurasia (Craig, 2003).

Hydatid cysts of *E. granulosus* develop in internal organs (mainly liver and lungs) of humans and intermediate hosts (herbivores such as sheep, horses, cattle, pigs, goats and camels) as unilocular fluid-filled bladders. These consist of a parasite-derived inner nucleated germinal layer and an outer acellular laminated layer surrounded by a host-produced fibrous capsule. Brood capsules and protoscoleces (PSC) bud off from the germinal membrane. Definitive hosts are carnivores such as dogs, wolves and foxes. Sexual maturity of adult *E. granulosus* occurs in the host small intestine within 4–5 weeks of ingesting offal containing viable PSC. Gravid proglottids or released eggs are shed in the faeces and, following their ingestion by a human or ungulate host, an oncosphere larva is released that penetrates the intestinal epithelium into the lamina propria. This is then transported passively through blood or lymph to the target organs where it develops into a hydatid cyst. Since the life cycle relies on carnivores eating infected herbivores, humans are usually a 'dead-end' for the parasite. Adult worm infections of *E. multilocularis* occur mainly in red and arctic foxes, although dogs and cats can also act as definitive hosts. Small mammals (usually microtine and arvicolid rodents) act as intermediate hosts. The metacestode of *E. multilocularis* is a tumor-like multivesicular, infiltrating structure consisting of numerous small vesicles embedded in stroma of connective tissue; the larval mass usually contains a semisolid matrix rather than fluid (Eckert & Plazes, 2004). CE and AE are

both serious diseases, the latter especially so, with a high fatality rate and poor prognosis if careful clinical management is not carried out.

In contrast to *E. multilocularis*, which appears to exhibit very limited genetic variation, an important feature of the biology of *E. granulosus* is that it comprises a number of intraspecific variants or strains that exhibit considerable variation at the genetic level. 10 distinct genetic types (genotypes G1–10) have been identified and this categorization follows very closely the pattern of strain variation emerging based on biological characteristics. The extensive variation in nominal *E. granulosus* may influence life cycle patterns, host specificity, development rate, antigenicity, transmission dynamics, sensitivity to chemotherapeutic agents and pathology with important implications for the design and development of vaccines, diagnostic reagents and drugs. A detailed account of genetic variation in *Echinococcus* and its implications can be found in (McManus, 2006), so this important topic will not be considered further here.

In our earlier review on the immunology and diagnosis of echinococcosis (Zhang *et al.*, 2003a), we considered immunity to infection in the intermediate and definitive hosts, innate resistance, evasion of the immune system, development of vaccines for use in intermediate and definitive hosts and, in particular, we emphasized procedures for diagnosis of CE and AE, including the value of immunodiagnostic and molecular approaches. Here we provide an update of recent progress in research on *E. granulosus* and *E. multilocularis,* especially highlighting advances in immunology, vaccine development, diagnosis and functional expression of key molecules.

Immunity to infection in the intermediate host

Although the host–parasite interplay, in most cases of echinococcosis appears to be harmonious and clinically asymptomatic for a long period after infection, the host does produce a significant immune response against the early stages of infection, while the parasite adapts highly effective evasive strategies to aid in survival.

Cystic echinococcosis (CE)

Clinical symptoms of CE reflect the presence of one or more unilocular fluid-filled cysts. About 70% of cysts are formed in the liver, followed by the lungs (20%), with the remainder involving other organs such as kidney, spleen, brain, heart and bone. Clinical manifestations are mild in the early stage, while as the cyst gradually grows, the parasite may physically damage tissues and organs, which can become dysfunctional at the later stages of echinococcosis. As discussed previously (Zhang *et al.*, 2003a), immune responses against early *E. granulosus* infection have mainly been investigated using experimental infections of mice and sheep. After the oncosphere locates a target organ, the small hydatid cyst that commences development is immediately confronted by the host immune responses, which are mainly cell-mediated, especially involving infiltration of macrophages and eosinophil cells, and low-level polarized Th1 responses. Antibody responses are weak and are, normally, undetectable in the early two to three weeks following infection.

There are extensive data on immune responses against the established cyst both from studies on patients with echinococcosis and from experimentally infected animals (Zhang *et al.*, 2003a). The established parasite produces significant quantities of antigens that modulate the immune responses and these include polarized Th2 responses, balanced with Th1 responses. The coexistence of elevated Th1 cytokines, especially interferon (IFN)-γ, and Th2 cytokines including IL-4, IL-5, IL-6 and IL-10, has been recorded in most hydatid patients where cytokine levels have been measured. In addition, IgG, especially IgG1 and IgG4, IgE and IgM are elevated as the cyst grows and becomes established. When a cyst dies naturally, is killed by chemotherapy treatment or is removed by surgery, Th2 responses drop rapidly, and Th1 responses become dominant. IgG levels can be maintained in humans for several years after the cyst has been removed. Once a patient suffers relapse, the Th2 responses regenerate very quickly.

In the early stages of echinococcal development, cellular responses may play a crucial role in protection against infection. A repeat challenge experiment showed that mice given a second oncospheral challenge 21 days after the primary infection with *E. granulosus* produced very high levels of protection (Zhang *et al.*, 2001) but with a very low antibody response (Zhang *et al.*, 2003d) at the time of the secondary challenge. Early experiments *in vitro* showed that neutrophils, in association with antibody, can bring about the killing of *E. granulosus* oncospheres (Rogan *et al.*, 1992), suggesting a possible role for antibody-dependent cell-mediated cytotoxicity (ADCC) reactions although antibody levels against this stage are low and/or cell-mediated immunity may induce killing. This is an important area that needs to be further explored as it may provide an understanding of the mechanisms of protection against the oncosphere with benefits for future vaccine design.

A remarkable feature of CE infection is the coexistence of both Th1 and Th2 responses. It is likely due to the presence on echinococcal antigens of distinct epitopes for each T-cell subset as it has been shown that a single recombinant protein can stimulate both types of response. The C-terminal region of a heat shock protein, Eg2HSP70, induced significantly greater amounts of tumor necrosis factor (TNF)-α, IFN-γ, and IL-10 in Eg2HSP70-stimulated peripheral blood mononuclear cells (PBMC) from CE patients compared with unstimulated cultures in all patients (Ortona

et al., 2003). Furthermore, the antigens EgA31 and EgTrp stimulated a significant amount of IL-12, IFN-γ, IL-10 and IL-6 cytokines in cytokine-producing splenocytes of BALB/c vaccinated with the two molecules compared with controls (Fraize *et al*., 2005).

The polarized T-cell responses are modulated by echinococcal antigens. Dematteis *et al*. (2003) analysed whether the cytokine responses in early and late experimental infection with *E. granulosus* depend on the dose of parasites to which the host is exposed. To this purpose BALB/c mice were inoculated intraperitoneally (i.p.) with either 500 or 2000 protoscoleces. Splenocytes of mice were obtained at days 3, 7, 14 and 21 and also on week 37 post-infection and were cultured *in vitro* with protoscolex antigens. Type-1 and type-2 cytokines were analysed in supernatants by enzyme-linked immunosorbent assay (ELISA). The lower number of protoscoleces induced an early type-0 cytokine response, whereas the inoculation of 2000 protoscoleces induced an early Th2 response. Parasite growth was lower in the group inoculated with the low infective dose, which stimulated type-0 cytokine responses that may be protective, while more protoscoleces may inhibit the protective Th0 or Th1 responses, invoking Th2 responses, which may be beneficial for parasite growth.

To further investigate the role of T lymphocytes in the immune response to *E. granulosus*, Rigano *et al*. (2004) generated T-cell lines from patients with active, transitional and inactive hydatid cysts and stimulated these using sheep hydatid fluid (SHF) and antigen B (AgB). The cell lines from a patient with an inactive cyst had a Th1 profile, while the T-cell lines derived from seven patients with active and transitional hydatid cysts had mixed Th1/Th2 and Th0 clones. The results showed that Th1 lymphocytes contribute significantly to the inactive stage of hydatid disease, with Th2 lymphocytes being more important in the active and transitional stages. This group (Rigano *et al*., 1995) had previously shown that, in human subjects undergoing pharmacological treatment with albendazole/mebendazole, a Th1 cytokine profile, rather than a Th2 profile, typically dominates, indicating that Th1 responses have a role in the process of cyst degeneration. An increased Th1-type cytokine IFN-γ response has been suggested as a marker for monitoring AE patient treatment (Dvoroznakova *et al*., 2004), whereas measurement of serum IL-4 may be a useful marker for the follow up of patients with CE (Rigano *et al*., 1999).

Ultrasound can be used to classify cysts into different clinical types according to the progression of cyst status (Wang *et al*., 2003). There are no data showing cytokine profiles associated with these cyst categories and this is clearly an area for future study. One aspect that is likely to be important is the influence of CD4+ T-helper lymphocytes on the control of such immunological mechanisms as they may impact on treatment (Vuitton, 2004). Self-cure is common for CE infection in sheep (Zhang & Zhao, 1992; Cabrera *et al*., 2003), and it most likely also happens in human populations in hyperendemic areas as patients with calcified cysts are often reported (Macpherson *et al*., 2004; Moro *et al*., 2005). Cytokines are likely to play a key role in the process of self-cure.

Hypersensitive immune responses/allergic reactions (urticaria, itching and anaphylactic shock) often complicate the course of CE. Some CE patients have elevated IgE levels to certain antigens, such as EgEF-1β/δ, AE21 and EgTeg, that appear to be associated with these allergic reactions (Colebrook *et al*., 2002; Ortona *et al*., 2003; Vuitton, 2004).

Alveolar echinococcosis (AE)

Human AE is a chronic and often fatal disease characterized by slowly developing cysts, mainly in the liver. Pathologically, 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 a viscous fluid, may form in the liver. Like CE infection, Th1 responses predominate in the early stages of AE infection, with the immune response switching to a Th2 polarized profile in later progression (Shi *et al*., 2003; Wei *et al*., 2004).

Another pathological characteristic of AE infection is the strong cellular immune response elicited by *E. multilocularis*. This results in a large granulomatous infiltrate surrounding the parasitic lesions (Vuitton *et al*., 1989; Ricard-Blum *et al*., 1996; Grenard *et al*., 2001), which is a process simular to granuloma formation and the resulting fibrosis associated with the pathology of schistosomiasis (Wynn *et al*., 2004). The cells involved in the formation of the periparasitic granuloma are mainly macrophages, myofibroblasts and T lymphocytes. In patients with abortive or dead lesions, a large number of CD4(+) T lymphocytes are present, whereas patients with active metacestodes display a significant increase in activation of predominantly CD8(+) T cells (Manfras *et al*., 2002), indicating that CD4(+) T cells play a role in the killing mechanism. This is supported by mouse experiments undertaken by Dai *et al*. (2004), who infected mice with *E. multilocularis* of different genetic backgrounds including micro MT, nude, T-cell receptor (TCR)-β(−/−), major histocompatibility complex (MHC)-I(−/−) and MHC-II(−/−) mice and found that at 2 months post-infection, the parasite mass was more than 10 times higher in nude, TCR-β(−/−) and MHC-II (−/−) mice than in infected C57BL/6 wild-type (WT) mice; furthermore these T-cell-deficient mice started to die of the high parasite load at this time-point. In contrast, MHC-I(−/−) and micro MT mice exhibited parasite growth rates similar to those found in WT controls. These findings clearly point to the major role that CD4(+) αβ(+)

T cells play in limiting *E. multilocularis* proliferation, while CD8(+) T and B cells appeared to play a minor role in the control of parasite growth. In the absence of T cells, especially CD4(+) or αβ(+) T cells, the cellular immune response to infection was decreased which resulted in the lack of hepatic granuloma formation around the parasite. In addition, in T-cell-deficient mice, the expression of IFN-γ and other inflammatory cytokines (except for interleukin-6) were increased in association with a high parasite load. Thus, the relative protection mediated by CD4(+) αβ(+) T cells against *E. multilocularis* infection seems not to be IFN-γ dependent, but rather relies on the effector functions of CD4(+) αβ(+) T cells (Dai *et al*., 2004).

The precise role that each of the cytokines play in fibrosis and development of AE lesions remains to be determined. Nevertheless, pretreatment of mice with interleukin (IL)-12 was extremely efficient in preventing the development of lesions and led to abortive parasitic vesicles surrounded by fully efficient periparasitic immune cell infiltration and fibrosis (Emery *et al*., 1998). Also, 75% of mice treated with IFN-α-2a had no hepatic lesions and half were fully protected. IFN-α-2a treatment markedly decreased the abnormally elevated production of IL-10 in both spleen cell cultures and peritoneal macrophage cultures from infected mice and restored phagocytosis and oxidative metabolism of macrophages. The treatment also inhibited IL-6 and IL-13 antigen-induced secretions in spleen cell cultures (Godot *et al*., 2003) and may be useful for treatment of AE patients; how these two Th1 cytokines may impact on the progression of AE is unknown but they likely act via inhibition of Th2 responses. IL-13 has clearly been shown to be a major factor in granuloma formation and the resulting fibrosis in schistosomal infection in the mouse model (Pearce & MacDonald, 2002), but there are no similar studies aimed at determining the role this cytokine plays in the hepatic fibrosis caused by AE.

Echinococcus multilocularis vesicle antigens have been shown to induce pro-inflammatory, regulatory and chemokine release by PBMC from patients (Eger *et al*., 2003). The pro-inflammatory cytokines IL-1β and IL-18 were reduced in echinococcosis patients; regulatory IL-10 was similar, but parasite vesicle-induced IL-8 was dominant and clearly elevated in patients. Such selective and opposite dynamics of inflammatory cytokines and chemokine release may prevent overwhelming and pathogenic inflammation, and constitute an appropriate response for attraction of effector cells into the periparasitic tissues with the capacity to limit *E. multilocularis* metacestode proliferation and dissemination.

The production of nitric oxide (NO) by intraperitoneal macrophages of mice during secondary infection with *E. multilocularis* mediates immunosuppression at the early and late stages of infection (Dai *et al*., 2003). NO production parallels the production of TNF-α (Shi *et al*., 2003) indicating that NO levels are enhanced by this cytokine. Antigens in the laminated-layer of the cyst decrease NO production *in vitro*, indicating that *E. multilocularis* produces molecules that can modulate the host immune responses (Andrade *et al*., 2004).

Antibody levels were shown to be low early on in murine (BALB/c) *E. multilocularis* infection but the levels of IgG1 and IgG3 increased significantly 8 weeks after challenge, and remained elevated throughout the 25 week period of observation (Shi *et al*., 2003). The production of IL-2R and TNF-α by spleen cells from infected mice stimulated with EmAg also increased significantly 8 weeks after infection, while IL-2R sharply decreased after 12 weeks of infection. During the period of 2–12 weeks after infection there was an increase in IL-1 secretion. The levels of IL-1 and TNF-α rapidly increased during the 16 weeks postinfection. A high level of IFN-γ was detected during the period of observation, and showed a peak at 12 weeks. These data indicate, as for *E. granulosus*, that Th1 is the major response in the early stage of infection, which is replaced by a Th2 response in the later phase of infection.

Extracts from metacestodes of *E. multilocularis* cause basophil degranulation, as well as the secretion of histamine, IL-4 and IL-13, in a dose-dependent manner. IgE stripping and resensitization of basophils indicating that the mechanism of IL-4 induction requires the presence of IgE on the cells (Aumuller *et al*., 2004). *Echinococcus multilocularis* may thus induce a Th2 response in their hosts by the induction of IL-4 release from basophils.

Vaccines and vaccination

The life-cycles of *E. granulosus* and *E. multilocularis* include two hosts: an intermediate and a definitive host. Effective CE control programs show that the prevention of transmission to either host can reduce or even eliminate the infection in human and livestock populations. Therefore, if either or both hosts can be vaccinated, the effect will be to improve and more rapidly expedite control. The sylvatic nature of the lifecycle of *E. multilocularis* makes a vaccination approach to control unlikely.

Vaccination of intermediate hosts

Substantial progress has been made towards developing a practical, recombinant vaccine (EG95) for use against *E. granulosus* in sheep (Gauci *et al*., 2005). The EG95 vaccine, cloned from the oncosphere, produces high levels of protection in terms of the reduction in the numbers of hydatid cysts in sheep, goats and cattle following experimental challenge in a number of countries, and natural challenge of sheep in China (Lightowlers & Heath, 2004). First described nearly a decade ago, the EG95 vaccine has,

however, yet to be introduced as part of any government-supported control program in areas endemic for hydatid disease. Economic and political factors will likely impede the large-scale use of the vaccine (Gauci *et al*., 2005). Further developments in recombinant protein production and in delivery of the EG95 vaccine may make it more amenable to widespread use. In this context, a commercial process was recently described for producing the recombinant vaccine in *E. coli* (Manderson *et al*., 2005). Furthermore, Marsland *et al*. (2003) described the construction of a recombinant orf (*Parapoxvirus*) virus expressing EG95 levels comparable to that achieved by a similar vaccinia virus recombinant. This recombinant virus will be a valuable tool with which to assess the potential of recombinant orf viruses to deliver vaccine antigens to sheep. If the virus can pass vertically from one generation to another, this would be a major step forward in the use of EG95 for the control of hydatid disease globally (Marsland *et al*., 2003).

Recent research indicates that the EG95 encoding gene belongs to a gene family of at least seven related genes, that at least some of the proteins encoded by the *eg95* gene family are expressed in other stages (immature and mature adult worms, protoscoleces) as well as in the oncosphere (Zhang *et al*., 2003b; Chow *et al*., 2004; Gauci *et al*., 2005). As described earlier, *E. granulosus* exhibits extensive strain variation, and variability of the *eg95* gene in different isolates of *E. granulosus* may directly impact the effectiveness of the EG95-based vaccine. Analysis of the *eg95* gene family from *E. granulosus* collected in Xinjiang, in northwest China, where hydatid disease is hyperendemic, showed the *eg95* gene family was shown to comprise two basic cDNA sequence types but very limited sequence variation was evident in the EG95 protein from oncospheres (Zhang *et al*., 2003b). This high degree of sequence conservation predicts that the vaccine will continue to be effective in China and elsewhere, although this needs to be fully substantiated, particularly should failures of the EG95 vaccine occur in the future.

A protein containing characteristic fibronectin III domains, related to EM95 (a homologue of EG95), that can induce significant levels of protection against challenge infection with *E. multilocularis* eggs in mice, has been identified in *E. multilocularis* (Merckelbach *et al*., 2003). Another recombinant *E. multilocularis* molecule, the 14–3–3 protein, elicited a high degree of protection (97%) against a primary egg challenge infection but no protection against secondary infection in vaccinated mice (Siles-Lucas *et al*., 2003); the results suggest the protective mechanisms were effective only against the oncosphere.

Vaccination of definitive hosts

In comparison with intermediate hosts of *Echinococcus*, immunization of canines has received very little attention in the past two decades. As the major definitive hosts for *E. granulosus*, dogs play a pivotal role in the transmission of hydatid disease. Interruption of the parasite life cycle in the dog host can provides a very acceptable and cost-effective complementary method for control by vaccination as there are far fewer dogs than sheep on farms so that fewer animals need vaccination and less vaccine doses would be necessary. The fact that old animals have a lower abundance and/or prevalence rates of *E. granulosus* compared with young dogs (Lahmar *et al*., 2001; Torgerson *et al*., 2003; Budke *et al*., 2005; Buishi *et al*., 2005; Moro *et al*., 2005) provides epidemiological evidence that canines may become resistant to reinfection in nature. Similar results have been described with *E. multilocularis* in naturally infected foxes (Hofer *et al*., 2000; Yimam *et al*., 2002; Losson *et al*., 2003). A mathematical model has indicated that there is significant herd immunity in dogs under a relatively high infection pressure (Torgerson, 2006).

It was found that when dogs were infected with *E. granulosus*, local and systemic specific antibodies and cellular responses were raised (Carmena *et al*., 2004, 2005; Moreno *et al*., 2004). While there was no relationship between serum IgA responses and parasite burden at the end of the infection, an inverse association of antiparasite IgE and parasite load appeared to exist. No differences were observed in the numbers of intestinal mast cells and goblet cells among all infected dogs (Moreno *et al*., 2004). Dogs infected with *E. multilocularis* also produced specific antibodies and T-cell responses to parasite antigens, although some modification of lymphocyte responses was apparent (Kato *et al*., 2005a), a situation also prevailing in the Mongolian gerbil, a prednisolone-untreated rodent definitive host model (Kato *et al*., 2005b).

In order to identify genes expressed only in mature adult worms that may encode targets for inhibiting egg production, thus forming the basis for developing a transmission blocking vaccine applicable to dogs, differences in mRNA expression between immature adult worms and mature adult worms of *E. granulosus* were determined using polymerase chain reaction-based differential display (DDRT-PCR) (Zhang *et al*., 2003c). As a result, examination of the deduced amino acid sequence of three of the corresponding complimentary DNAs (cDNAs) (egM4, egM9 and egM123) indicated they were cysteine-rich and contained a 24 amino acid repeat sequence, repeated four to six times. The repeat regions were predominantly α helical in nature with interspersed turns, forming alternating zones of positive and negative charge. The functional significance of each of the cDNAs identified was unclear as none had significant sequence similarity to genes of known function. Nevertheless, polypeptides encoded by egM4 and egM123 were recognized by antibodies in a serum pool from dogs experimentally infected with *E. granulosus*, suggesting they

could prove of value in serodiagnosis of definitive hosts. In addition, pilot vaccine/challenge experiments in dogs with proteins encoded by egM4, egM9 and egM123 showed encouraging results in terms of reduced egg production (Zhang, 2003), suggesting they may prove of value as components of a vaccine effective in the definitive host.

Diagnosis

Early diagnosis of CE and AE can provide significant improvements in the quality of the management and treatment of both diseases. In most cases, the early stages of infection are asymptomatic, so methods that are relatively easy to use and that are cheap are required for large-scale epidemiological surveillance of populations at high risk. Immunodiagnosis provides such an approach and can, additionally, confirm clinical findings. The definitive diagnosis for most human cases of CE is by physical imaging methods, such as radiology, ultrasonography, computed axial tomography (CT scanning) and magnetic resonance imaging although such procedures are often not readily available in isolated communities. Immunodiagnosis can also play an important complementary role. It is useful not only in primary diagnosis but also for follow-up of patients after surgical or pharmacological treatment. Additional advantages of immunodiagnosis include screening of large populations in communities from endemic areas, rapid testing of individuals in remote areas where imaging equipment may not be readily available, for follow-up monitoring of subjects in endemic areas, and for confirmation of CE or AE cases when physical imaging does not provide a definitive diagnosis (see Qaqish *et al*., 2003; Hernandez *et al*., 2005). Immunodiagnosis can also play a major role in the detection of AE infection, which is very important for early commencement or treatment, because of the high associated mortality.

CE serodiagnosis

The detection of circulating *Echinococcus granulosus* antigens in sera is less sensitive than antibody detection, which remains the method of choice. CE serology has a very long history and almost all serological tests that have been developed have been used in the diagnosis of human cases. 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 tests have been replaced by the ELISA and immunoblotting (IB) in routine laboratory applications (see, e.g. Nasrieh & Abdel-Hafez, 2004) although the choice of diagnostic antigens exhibiting the requisite specificity and sensitivity is ever critical.

The lipoproteins antigen B (AgB) and antigen 5 (Ag5), the major components of hydatid cyst fluid (HCF), have been the two molecules that have received wide attention in regards to diagnosis. Along with HCF, they are the most widely used antigens in current assays for immunodiagnosis of CE. Both antigens have been well characterized by immunoblotting and/or by immunoprecipitation of radiolabeled antigen and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). 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.

Antigen B

Antigen B is a polymeric lipoprotein with a molecular weight of 120 kDa. It can be measured in patient blood as circulating antigen and it has been suggested that AgB has an important role in the biology of the parasite and its relationship with the host. AgB is a highly immunogenic molecule, a characteristic that underpins its value in serodiagnosis. It appears ladder-like under reduced condition on SDS-PAGE with three bands (subunits AgB1, AgB2 and AgB3) with molecular sizes of approximately 8 or 12, 16 and 24 kDa, suggestive that it comprises polymers of 8 kDa subunits. The smallest subunit has proved the most useful target in diagnostic studies. A possible new AgB subunit (AgB4) was recently identified (Arend *et al*., 2004) and recent work shows that AgB is encoded by a multigene family (Haag *et al*., 2006). Furthermore, AgB presents homology to, and shares apparent structural similarities with, helix-rich hydrophobic ligand binding proteins (HLBPs) from other cestodes, and has fatty acid binding properties (Chemale *et al*., 2005).

Two residues in the AgB sequence are critical for diagnosis (Gonzalez-Sapienza & Cachau, 2003). The N-terminal extension of the major subunit of AgB concentrates the immunoreactive B-cell epitopes of the native molecule. The nature of this immunodominance was analyzed using four monoclonal antibodies (mAbs) defining overlapping epitopes in this region of the AgB molecule. The minimal epitope requirements of these mAbs were determined using phage display peptide libraries. The consensus sequences isolated with the mAbs, and alanine replacement analysis with synthetic peptides mapped the relevant molecular contacts within a short stretch corresponding to residues 17–24 of the AgB major subunit. Substitution of two critical residues within this stretch produced a dramatic loss of antigenicity, as determined using patient sera.

To date, several AgB cDNAs have been cloned, expressed as recombinant proteins and used for diagnosis; in addition, a number of AgB peptides have been synthesized and used in ELISA for diagnostic purposes. 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. Two of the recombinant subunit components (rAgB8/1 and rAgB8/2) of AgB have been used widely in diagnosis. Virginio *et al.* (2003) showed that, of six purified recombinant proteins tested in ELISA for specific IgG with a panel of sera from patients with surgically confirmed or immunologically diagnosed CE, AgB8/2 provided the highest diagnostic sensitivity (93.1%) and specificity (99.5%). Furthermore, different IgG isotypes showed dominance in the response for each of the recombinant antigens. There was a clear predominance of IgG4 response for all antigens tested, indicating that this would be the subclass of choice to be assessed for these, and possibly other, recombinant proteins. The detection by immunoblotting of antibodies specific for the 8 kDa subunit of antigen B and in particular the IgG4 subclass expression, has also been advocated by Siracusano *et al.* (2004) as a promising serodiagnostic tool.

To compare variability between laboratories, six South American groups double-blind tested six antigens against the same serum collection (Lorenzo *et al.*, 2005). High intercenter reproducibility was attained and the results showed that HCF, native AgB and its recombinant AgB8/1 subunit had almost the same efficiency at 81.4, 81.3 and 81.9%, respectively, with the diagnostic efficiencies for an AgB-derived synthetic peptide peptide, the recombinant AgB8/2 subunit, and recombinant cytosolic malate dehydrogenase from *E. granulosus* (EgMDH) being less efficient at 76.8, 69.1 and 66.8%, respectively. Different regional batch preparations of HCF yielded different diagnostic performances but the group recommended that recombinant AgB should be used as the standard antigen in laboratory analysis.

Using a novel approach, Chen *et al.* (2003) generated a genetically engineered antibody against recombinant AgB that may have potential implications in immunological treatment and drug targeting delivery.

Antigen 5

There have been few studies on Ag5 in recent years. Ag5 is a very high molecular weight (*c.* 400 kDa) lipoprotein complex composed of 57 and 67 kDa components that under reducing conditions dissociate into 38 and 22–24 kDa subunits. 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. Although they did not carry out any serodiagnostic studies, Lorenzo *et al.* (2003a) cloned the Ag5 gene by reverse transcription-PCR on the basis of the amino acid sequences of tryptic fragments. The nucleotide sequence indicated that Ag5 is synthesized as a single polypeptide chain that is afterwards processed into single disulphide-bridged 22 and 38 kDa subunits. Whereas the 22-kDa component contains a highly conserved glycosaminoglycan-binding motif that may help to confine Ag5 in the host tissue surrounding the parasite, the 38 kDa subunit is closely related to serine proteases of the trypsin family (Lorenzo *et al.*, 2003a). However, neither proteolytic activity nor binding to protease inhibitors could be detected using native purified Ag5. Thus it may be possible that Ag5 possesses a highly specific physiological substrate or, more likely, that trypsin-like folding has been recruited to fulfil novel functions. Subsequently, this group (Lorenzo *et al.*, 2005) prepared two recombinant forms of the antigen, namely, rAg5 (corresponding to the unprocessed polypeptide chain of the antigen) and rAg5–38s (corresponding to its 38 kDa subunit). Their antigenicities were compared to that of the native antigen using a human serum collection. There was a major drop in the reactivity of the sera, particularly against rAg5–38s, which was confirmed by analysis of the cross-reactivity of two panels of monoclonal antibodies specific for rAg5–38s and the native antigen. Using the chemically deglycosylated native antigen, these authors demonstrated that the reduced antigenicity of the recombinants was due to the loss of the sugar determinants, and not to their misfolding. Inhibition experiments using phosphorylcholine confirmed that this moiety also contributes to the reactivity of the antigen, but to a much lesser extent. The presence of immunodominant highly cross-reactive glycan moieties in the Ag5 molecule may involve a parasite evasion mechanism, as was earlier shown for AgB (see Zhang *et al.*, 2003a).

Studies by Mahmoud & Abou Gamra (2004) have shown that a purified alkaline phosphatase (EgAP) extracted from *E. granulosus* hydatid cyst membranes possesses 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. This mirrors the diagnostic value previously shown for purified alkaline phosphatase (pAP) from *E. multilocularis* metacestodes (see Zhang *et al.*, 2003a).

Other antigens and approaches for immunodiagnosis

In a significant advance, Li *et al.* (2003) used a pool of serum samples from mice infected with oncospheres (eggs) of *E. granulosus* to screen a cDNA library constructed with RNA extracted from protoscolex larvae from sheep hydatid cysts. One immunoreactive clone, designated EpC1, was shown to encode a protein of 76 residues. The cDNA fragment was

subcloned into an expression vector, pET-41b(+), and the resulting recombinant EpC1 glutathione S-transferase (GST) fusion protein (rEpC1–GST) was expressed in *Escherichia coli* and was affinity purified against the GST tag. Immunoglobulin G was the dominant antibody isotype generated against rEpC1–GST. A total of 896 human serum samples were used to evaluate the diagnostic sensitivity and specificity of the fusion protein by immunoglobulin G immunoblotting; 324 serum samples from patients with CE, 172 from patients with neurocysticercosis, 89 from patients with alveolar echinococcosis, and 241 from patients with other infections or clinical presentations, as well as 70 from confirmed-negative control subjects, 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 unprecedented, taking into account the large panel of serum samples that were tested (Table 1).

In addition to testing recombinant antigen B subunits, Virginio *et al.* (2003) assessed the diagnostic potential in ELISA of 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 261370) of an actin filament fragmenting protein. These recombinant antigens yielded sensitivities between 58.6 and 89.7%, and three of them were considered of complementary value.

Purified recombinant thioredoxin peroxidase of *E. granulosus* (TPxEg) was used to screen sera from heavily infected mice and patients with confirmed hydatid infection. 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 (Li *et al.*, 2004).

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 western blotting or ELISA.

Application of serodiagnosis for evaluation of infection risk in populations

An important area for study is the evaluation of the diagnostic potential of serodiagnostic procedures in the field. Qaqish *et al.* (2003) used ELISA to determine the seroprevalence of CE in different communities in Jordan. The rural-agricultural subjects were significantly more likely to be seropositive (11.4%) than the semi-Bedouin (5.0%) or Bedouin (3.7%).

In another study, Hernandez *et al.* (2005) screened villagers in an area with high prevalence of CE in rural Tacuarembo, Uruguay. The correlation between serological data and the incidence of risk factors carried out on 480 individuals who were examined by means of abdominal sonography (local prevalence = 0.8%). Serum samples (305) were analysed by ELISA to determine specific IgG against crude antigens from *E. granulosus*. A total of 27 individuals exhibiting no detectable changes in abdominal sonographic examination were found to be seropositive ('ultrasound normal group'). Of these individuals, nine were seroreactive against purified AgB. A significant degree of correlation was found between seroreactivity and the incidence of some risk factors (CE antecedent in the family, $P < 0.005$ and use of rural water, $P < 0.0001$) among this group. Follow-up of individuals of the 'ultrasound normal group' was carried out after 2 years to evaluate the implications of this serological reactivity. No predictive value for cyst development was assessed with complementary image study; in contrast

Table 1. Performance comparison of recombinant EpC1-GST protein with native hydatid cyst fluid antigen B (HCF-AgB) for human cystic echinococcosis (CE) serodiagnosis by immunoblotting (data summarized from Li *et al.* 2003, 2004)

Disease/clinical presentation	Number of serum samples	Positive serum samples; number (%)		
		EpC1-GST	GST	HCF-AgB
CE, before surgery	116	107 (92.2)	5 (4.3)	98 (84.5)
AE	89	4 (4.5)	2 (2.2)	46 (52)
Taenia solium NCC	172	16 (9.3)	7 (4.1)	14/100 (14)*
Schistosomiasis	78	1 (1.3)	1 (1.3)	5/50 (10)*
Other parasitic infection	116	1 (0.9)	1 (0.9)	4/72 (5.6)*
Non-infectious diseases	47	0	1 (3.7)	1 (3.7)
Healthy controls	70	3 (4.3)	1 (1.4)	3 (4.3)

*Partially tested.

CE, cystic echinococcosis; AE, alveolar echinococcosis; NCC, neurocysticercosis.

transient antibodies were observed with both crude and purified antigen as approximately 60% of individuals became negative when re-sampled. This study showed that serodiagnostic data can prove useful for evaluating the efficacy of CE control efforts.

Deutz *et al.* (2003) investigated the seroprevalence of a range of zoonotic pathogens, including *E. granulosus* and *E. multilocularis*, in hunters originating from the south-eastern Austrian federal states of Styria and Burgenland, and compared the results with other predisposed occupational groups. The high seroprevalences for CE and AE, in addition to several other pathogens, demonstrated that hunters are particularly exposed to zoonotic pathogens, which is perhaps not surprising giving their occupation and lifestyle (Deutz *et al.*, 2003).

Challenges for immunodiagnosis

Despite the development of sensitive and specific techniques, such as immunoblotting and ELISA, the immunodiagnosis of CE in clinical practice and population studies remains a complex task and three major problems still remain. The first problem is that most available screening tests give a high percentage of false negative results which can be as much as 50% with sera taken from communities in population surveys (Moro *et al.*, 2005). To explore possible factors associated with false negative antibody response in immunodiagnosis of CE patients, Xu *et al.* (2002) determined IgG subclasses (IgG1, IgG2, IgG3, IgG4) and IgA, IgM and IgE in the sera of individuals with a negative total IgG response; they concluded that the seronegative response of total IgG in CE patients might be due to low levels of specific IgG, variant Ig antibody expression and/or formation of circulating immune complexes, and that the combined detection of IgG1+IgA+IgM could enhance the sensitivity of serological tests in CE patients (Xu *et al.*, 2002).

The second issue relates to putative false positive reactions, the causes for which may be very complex. Cross-reactivity with antibodies from other infections may be one reason for this false-positivity, but it may also be due to the fact that CE antibodies can remain in serum for long periods following surgical removal or effective drug treatment of cysts, or even if the infection self-cures as discussed earlier.

The third problem is how to distinguish active or progressive cases of echinococcosis from cured individuals. It has been shown in a number of studies that IgG, especially IgG1 and IgG4, can remain circulating in the human blood system for more than 5 years (Bulut *et al.*, 2001; Li *et al.*, 2003; Nasrieh & Abdel-Hafez, 2004), with IgA and IgM also detectable in serum 3 years after surgical cyst removal (Doiz *et al.*, 2002). In addition, significantly high levels of specific IgE serum antibodies determined by ELISA were still detectable one year after surgery (Bulut *et al.*, 2001).

In order to address this issue, Lawn *et al.* (2004) investigated whether concentrations of CE-specific IgG subclasses 1–4 in ELISA using crude horse hydatid cyst fluid as an antigen correlated better with disease activity than total IgG. They studied a cohort of patients with symptomatic CE treated with anthelminthic drugs and surgery and who were followed up clinically and radiologically for several years (Lawn *et al.*, 2004). Changes in concentrations of antibodies were correlated with clinical and radiologic outcome. At diagnosis, concentrations of CE-specific total IgG, IgG1 and IgG2 antibodies were significantly elevated in a greater proportion of patients compared with IgG3 and IgG4 antibodies. The fact that using cyst fluid antigen rather than purified antigen (AgB or Ag5) yielded better responses could be that crude antigens enhance detection of subclass antibodies with different antigen specificities with IgG2 antibodies being most sensitive. Importantly, during post-treatment follow up, the IgG2 antibody response provided the best correlate of disease activity, with serum concentrations of CE-specific antibodies showing that IgG2 correlated most strongly with clinical outcome (Lawn *et al.*, 2004).

Immunodiagnosis of CE in animal intermediate hosts

In comparison with 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. Accurate serological diagnosis of CE infection in livestock is difficult due to serological cross-reactions with several other species of taeniid cestodes including *Taenia hydatigena* and *Taenia ovis*. 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. In sheep, the principal intermediate host of *E. granulosus* in most endemic regions of the world, antibodies to various antigens including antigen 5 are detectable in the sera of some, but not all infected sheep ('nonresponders'). As with human CE, the detection of circulating antigen does not appear to be useful for diagnostic purposes.

Ibrahem *et al.* (2002) used AgB, partially purified from hydatid cyst fluid from camels or sheep, and a recombinant form of AgB (r-AgB) in an ELISA, to screen panels of serum samples from slaughtered camels and sheep naturally infected with CE (Ibrahem *et al.*, 2002). Seroreactivity, however, was variable. Native AgB gave the highest sensitivity (97%) in ELISA for camel CE. In contrast, r-AgB gave lower sensitivity for camel (84%) and sheep (28%) CE. The r-AgB-ELISA was, however, highly specific, yielding 90 and 95%

specificity, respectively, for natural camel and sheep CE infections (Ibrahem *et al*., 2002).

Kittelberger *et al*. (2002) carried out a very extensive study aimed at developing an immunological method for the identification of sheep infected with *E. granulosus* which would allow the monitoring of animals imported into countries free from hydatidosis, and as an aid to countries where control schemes for the disease are in operation. Three ELISAs were developed and validated, using as antigen purified 8 kDa AgB hydatid cyst fluid protein (8kDaELISA), recombinant EG95 oncosphere protein (OncELISA) or a crude protoscolex preparation (ProtELISA). Sera used for the assay validations were obtained from 249 sheep that were 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%, the assay would be useful for the detection of the presence of infected sheep on a flock basis but not for reliable identification of individual animals infected with *E. granulosus* (Kittelberger *et al*., 2002).

In a later study, Simsek & 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 & Koroglu, 2004).

AE serodiagnosis

The diagnosis of AE is based on similar findings and criteria as in CE. These include case history, clinical findings, morphological lesions identified by imaging techniques, PCR or immunofluorescence/immunohistochemistry, and immunodiagnosis. Like CE, serodiagnosis of alveolar echinococcosis provides a complementary role to other procedures in early detection of the infection. The methods are similar to those used for CE but serological tests for antibody detection are generally more reliable. As we have emphasized earlier, AE is a very serious disease with a high fatality rate, so early detection is paramount in order that successful management and treatment can commence.

Em2, a species-specific native antigen isolated from the metacestode of *E. multilocularis* (Gottstein, 1992) has been used successfully over a long period for immunodiagnosis of human AE; the sensitivities of Em2 with ELISA vary depending upon the geographical origin of the patient, ranging between 77 and 92%. In addition, serology for antibodies against the Em2 antigen have been shown to be a useful method for identifying animals including cynomolgus mokeys (*Macaca fascicularis*) (Bacciarini *et al*., 2004; Rehmann *et al*., 2005) and lowland gorillas (*Gorilla g. gorilla*) (Rehmann *et al*., 2003) that might be infected with *E. multilocularis* and are therefore at risk of developing fatal AE.

The Em2plus ELISA, a combination of Em2 with a recombinant protein designated II/3–10 (also termed EM10), increased the sensitivity to 97% (Gottstein *et al*., 1993). The Em2plus assay exhibits cross-reaction with CE (in 25.8% of cases) which is higher than the individual Em2 (5.6%) and 11/3–10 (6.5%), but limited cross-reactivity with other diseases. The Em2plus-ELISA has been commercialized for clinical diagnosis of AE and for population screening (Gottstein *et al*., 1993).

EM10 (Em II/3–10) shares almost complete identity to the *E. granulosus* protein sequences EG10 and EGII.3 (Sako *et al*., 2002). Although the two species have similar sequences, recombinant EM10 protein showed very high specificity to distinguish AE infection from CE infection in human patients. It is not clear why these homologous molecules exhibited such a different pattern of sero-recognition. Sako *et al*. (2002) suggested that EG10 may be expressed at a very low level in larval *E. granulosus*, but it may equally be that the transcription of *EG*10 is low or silenced. An 18 kDa antigen (Em18) from protoscoleces of AE was reported as being a highly species-specific (96.8%) and sensitive (97%) antigen with potential not only for differentiation of AE from either CE or other helminth infections, but also for differentiation of active from inactive AE (Ito *et al*., 1995; Akira, 1997). Subsequently, EM18 was shown to be a fragment of the C-terminal of EM10 and the recombinant protein was recognized by 87.1 and 90.3% of 31 serum samples from AE patients in ELISA and immunoblotting, respectively (Sako *et al*., 2002). Recombinant Em18-ELISA andEm18-immunoblot assays have proved invaluable for differentiating AE from CE infection (Ito *et al*., 2002), the former also being useful for evaluating the efficacy of treatment in patients with AE (Fujimoto *et al*., 2005). Epitope mapping indicated that the part of the ReEm18 antigen sequence necessary for AE diagnosis occurs in the N-terminal half to two-thirds of the entire sequence (Jiang *et al*., 2004).

Full-length cDNA and genomic DNA encoding an 8-kDa subunit of antigen B from *E. multilocularis* (designated EmAgB8/1) were isolated from an *E. multilocularis* metacestode cDNA library and a protoscolex genomic DNA library, respectively (Mamuti *et al.*, 2004). Reverse transcription-PCR analysis revealed that the clone encoding EmAgB8/1 is predominantly transcribed in larval *E. multilocularis*. The mature form was expressed in *Escherichia coli*, and its antigenic reactivity was compared with that its counterpart 8 kDa subunit of *E. granulosus* AgB (EgAgB8/1), by Western blotting and ELISA with serum samples from patients confirmed to have CE and AE. The sensitivity of EmAgB8/1 was comparable to that of EgAgB8/1 for the serodiagnosis of echinococcosis. It is noteworthy that there was no cross-reaction with sera from patients with cysticercosis, which often cross-react when native antigens are used for serodiagnosis.

Direct detection or amplification of *E. multilocularis* nucleic acids in clinical samples is also considered useful for the primary diagnostic identification of parasite materials in biological specimens resected or biopsied from patients, and also for the assessment of the viability of parasite samples after chemotherapy or other treatment. Infections of humans with *E. multilocularis*, have been described with increasing frequency in Poland since 1994. In the attempt to verify these reports, specimens were obtained from a group of Polish patients (Myjak *et al.*, 2003). Liver lesions in patients with AE, diagnosed on the basis of results of histological and serological tests, were shown, by the presence of specific microsatellite sequences and mitochondrial 12S rDNA, to contain *E. multilocularis* DNA. These data provided unequivocal proof that human infections with *E. multilocularis* occur in Poland. PCR-amplification of specific *E. multilocularis* DNA has proved useful also for helping to diagnose unambiguously AE infection in monkeys (Bacciarini *et al.*, 2004).

Diagnosis of echinococcosis in definitive hosts

The detection of *Echinococcus* infections in canines is important for epidemiological surveillance and evaluation of echinococcosis control programs. Diagnosing *Echinococcus* infections in dogs and other definitive hosts is problematical as the eggs of taeniid cestodes are extremely similar, and thus identification by microscopic examination of the faeces is risky and non-specific. Two major diagnostic methods have been extensively used in dogs, purgation with arecoline compounds and necropsy of the small intestine. Necropsy is the method of choice for foxes and other final hosts. Two immunodiagnostic approaches have been developed for diagnosis of *E. granulosus* and *E. multilocularis* infection in definitive hosts – assays for specific serum antibody and detection of parasite products (coproantigens) in faeces. Overall, the available ELISA-based methods for detection of circulating antibodies in canines have poor sensitivity, specificity is unclear and there is no correlation with worm burden so their usefulness, other than in population-based studies of canine hosts, is questionable (Zhang *et al.*, 2003a).

Much more success has been achieved using the other major approach to immunological diagnosis of *Echinococcus* infection in the definitive host by detection of adult worm products in faeces using sandwich ELISA methodology and this technique has been widely used with successful detection of *E. granulosus* and *E. multilocularis* (Lopera *et al.*, 2003; Sanchez Thevenet *et al.*, 2003; Benito & Carmena, 2005; Cavagion *et al.*, 2005; Moro *et al.*, 2005; Reiterova *et al.*, 2005). Although these tests for diagnosis of canine echinococcosis provide high specificity and sensitivity, little is known of the characteristics of antigenic molecules present in faeces from infected animals. While initial attempts to determine the molecular weights of *E. granulosus* coproantigens by SDS-PAGE and Western blotting with coproantigen reactive capture antibodies were equivocal, they suggested the presence of a significant carbohydrate component. Elayoubi *et al.* used SDS-PAGE and western blot to show that the coproantigens are highly glycosylated and contain β-galactose and *N*-acetyl-β-glucosamine (Elayoubi *et al.*, 2003). Subsequently, supernatants prepared from *E. granulosus*-infected dog faecal samples and fractionated by size-exclusion fast protein liquid chromatography (FPLC) showed that the antigens are large molecular weight molecules that may be derived from the carbohydrate-rich surface glycocalyx of adult worms, and are shed, released or secreted during the life-span of the tapeworm (Elayoubi & Craig, 2004).

Copro-antigen detection allows *in vitam* diagnosis of *Echinococcus* infections but, as well, several PCR-based protocols have been developed that allow identification of *Echinococcus* DNA from eggs or from adult parasites. These provide a highly complementary approach for positive and highly specific diagnosis of canines infected with *E. granulosus* and *E. multilocularis* (Cabrera *et al.*, 2002; Abbasi *et al.*, 2003; Deplazes *et al.*, 2003; Casulli *et al.*, 2004, 2005; Stefanic *et al.*, 2004; Varcasia *et al.*, 2004; Reiterova *et al.*, 2005) and environmental detection of *Echinococcus* eggs in soil samples (Shaikenov *et al.*, 2004).

The efficacy of two PCR-based methods to detect patent and prepatent infection in dogs experimentally infected with *E. granulosus* was recently compared (Naidich *et al.*, 2006). The detection was based on amplification of a fragment of the mitochondrial *cox1* gene (Mit-PCR) and a repetitive element (Rep-PCR) from the genome of *E. granulosus*. The ability of both methods to detect several genotypes of the parasite were assessed. Both PCR methods could detect *E. granulosus* during both prepatent and patent periods, even

when microscopical observation of eggs in faecal samples was negative. The Mit-PCR produced the same amplification pattern for all the parasite genotypes tested while the amplification patterns with the Rep-PCR differed among groups of strains. In addition, faecal samples collected from dogs from an endemic area in Argentina were diagnosed with more sensitivity using the PCR methods than by arecoline hydrobromide purgation. These copro-PCR tests can thus be applied in the confirmation of coproantigen-positive fecal samples and to verify the success of control programs.

Outlook: genomics and the functional expression of genes from *Echinococcus*

A series of recent studies have investigated the functional expression of genes from *E. granulosus* and *E. multilocularis*. All parasitic helminths, including *Echinococcus* process a large subset of their mRNA molecules via spliced leader trans-spling (Fernandez *et al*., 2002). In an important contribution, Brehm *et al*. (2003) described a rapid and efficient method, 'spliced leader differential display', to analyse gene expression patterns of *in vitro* cultivated protoscoleces and metacestode vesicles from *E. multilocularis*. The approach was further used to identify genes which are induced in the *E. multilocularis* metacestode stage under growth-promoting conditions in the presence of host hepatocytes. One mRNA, encoding a putative member of the epidermal growth factor family of mitogens, was shown to be 10-fold induced upon co-incubation of metacestode vesicles with host cells which indicated a possible involvement of parasite-determined, epidermal growth factor-like signal transduction systems in metacestode proliferation and development. Shortly thereafter, an *E. multilocularis* candidate receptor for Egfd was identified by Spiliotis *et al*. (2003). The identified molecule, EmER, displayed clear homologies to EGF receptors from different phylogenetic origins and was expressed in metacestode and protoscolex. It has not yet been experimentally shown that Egfd and EmER form a corresponding ligand–receptor pair. However, the fact that *emer* is the only *E. multilocularis* gene that encodes an EGF receptor strongly suggests that this is the case.

In another approach, Rosenzvit *et al*. (2006) used the signal sequence trap technique to identify genes coding for secreted and membrane-bound proteins from *E. granulosus*. A number of isolated clones showed significant similarity with amino acid transporters, Krebs cycle intermediates transporters, presenilins and vacuolar protein sorter proteins. Other cDNAs encoded secreted proteins without homologues. All the mRNAs were expressed in protoscoleces and adult worms, but some of them were not found in oncospheres. These identified putative *E. granulosus* secreted and membrane-bound proteins are likely to play important roles in the metabolism, development and survival of the parasite in the host.

A series of more conventional studies have been undertaken on the cloning, characterization and functional expression of a number of molecules from both *E. granulosus*

Table 2. Examples of molecules isolated from *Echinococcus* and their function

Molecule	Main function	Reference
Ras and Raf	Regulatory factors	Spiliotis *et al*. (2005)
EmSkip	SNW/SKIP transcriptional coregulator	Gelmedin *et al*. (2005)
SNW/SKIP	Transcriptional co-regulators	Gelmedin *et al*. (2005)
Thioredoxin peroxidase	An element in the thioredoxin system	Li *et al*. (2004)
Ral-like GTPases	Binding and hydrolyzing guanosine triphosphate (GTP)	Spiliotis & Brehm (2004)
PDZ-1	NERMAD-specific interaction partner of the ERM protein	Hubert *et al*. (2004)
Mitochondrial malate dehydrogenase (mMDH)	Krebs cycle enzyme	Aguero *et al*. (2004)
UDP-*N*-acetyl-D-galactosamine:polypeptide *N*-acetylgalactosaminyltransferase	Biosynthesis of *O*-glycosylated proteins	Freire *et al*. (2004)
EmSmad	Smad family, important role in transforming growth factor signaling cascade	Zavala-Gongora *et al*. (2003)
Thioredoxin glutathione reductases	Reducing thioredoxin and glutathione in the antioxidation system	Agorio *et al*. (2003)
Tyrosine kinase	Phosphorylation	Konrad *et al*. (2003)
EmIR	Insulin receptor, signaling	Konrad *et al*. (2003)
Ag5	Proteases of the trypsin family	Lorenzo *et al*. (2003b)
EgAFFP	Actin filament fragmenting and nucleating activities	Cortez-Herrera *et al*. (2001)
H-FABPs	Heart group of fatty acid binding protein	Alvite *et al*. (2001)
Egact	Actin, promoting transcription	Gimba *et al*. (2000)
EM10	Ezrin–radixin–moesin (ERM)	Hubert *et al*. (1999)

and *E. multilocularis* which include regulatory factors, signalling factors, receptors and enzymes (Table 2).

Currently, genomic and expressed sequence tag (EST) information on *Echinococcus* spp. is very limited compared with studies on the filaria and schistosomes although plans are in place to produce 60 000 ESTs to characterize genes from *E. granulosus* and *E. multilocularis* metacestodes (http://www.sanger.ac.uk/Projects/Echinococcus/). This new information will add significantly to the available *Echinococcus* genomic and cDNA sequences in GenBank and the current collection of >3000 EST clusters for trans-spliced and non-trans-spliced cDNAs of *E. granulosus* and *E. multilocularis* available on the LophDB search base (http://zeldia.cap.ed.ac.uk/Lopho/LophDB.php), established by John Parkinson (Toronto, Canada).

The new developments in genomics, proteomics (Chemale *et al.*, 2003) and microarray analysis that are underway or planned for the *Echinococcus* organisms will underpin future advances on the functional biology of these important, emerging (Eckert & Deplazes, 2004) pathogens, provide a better understanding of the parasite–host interplay in echinococcal infections, and new avenues for the identification of additional targets for diagnosis, vaccination and chemotherapy.

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References

Abbasi I, Branzburg A, Campos-Ponce M, Abdel Hafez SK, Raoul F, Craig PS & Hamburger J (2003) Copro-diagnosis of *Echinococcus granulosus* infection in dogs by amplification of a newly identified repeated DNA sequence. *Am J Trop Med Hyg* **69**: 324–330.

Agorio A, Chalar C, Cardozo S & Salinas G (2003) Alternative mRNAs arising from trans-splicing code for mitochondrial and cytosolic variants of *Echinococcus granulosus* thioredoxin Glutathione reductase. *J Biol Chem* **278**: 12920–12928.

Aguero F, Noe G, Hellman U, Repetto Y, Zaha A & Cazzulo JJ (2004) Purification, cloning, and expression of the mitochondrial malate dehydrogenase (mMDH) from protoscolices of *Echinococcus granulosus*. *Mol Biochem Parasitol* **137**: 207–214.

Akira I (1997) Serodiagnosis of alveolar echinococcosis: detection of antibody against EM18 in patients and rodents. *Southeast Asian J Trop Med Public Health* **28**: 117–124.

Al-Sherbiny MM, Farrag AA, Fayad MH, Makled MK, Tawfeek GM & Ali NM (2004) Application and assessment of a dipstick assay in the diagnosis of hydatidosis and trichinosis. *Parasitol Res* **93**: 87–95.

Alvite G, Di Pietro SM, Santome JA, Ehrlich R & Esteves A (2001) Binding properties of *Echinococcus granulosus* fatty acid binding protein. *Biochim Biophys Acta* **1533**: 293–302.

Andrade MA, Siles-Lucas M, Espinoza E, Perez Arellano JL, Gottstein B & Muro A (2004) *Echinococcus multilocularis* laminated-layer components and the E14t 14–3–3 recombinant protein decrease NO production by activated rat macrophages in vitro. *Nitric Oxide* **10**: 150–155.

Arend AC, Zaha A, Ayala FJ & Haag KL (2004) The *Echinococcus granulosus* antigen B shows a high degree of genetic variability. *Exp Parasitol* **108**: 76–80.

Aumuller E, Schramm G, Gronow A, Brehm K, Gibbs BF, Doenhoff MJ & Haas H (2004) *Echinococcus multilocularis* metacestode extract triggers human basophils to release interleukin-4. *Parasite Immunol* **26**: 387–395.

Bacciarini LN, Gottstein B, Pagan O, Rehmann P & Grone A (2004) Hepatic alveolar echinococcosis in cynomolgus monkeys (Macaca fascicularis). *Vet Pathol* **41**: 229–234.

Benito A & Carmena D (2005) Double-antibody sandwich ELISA using biotinylated antibodies for the detection of *Echinococcus granulosus* coproantigens in dogs. *Acta Trop* **95**: 9–15.

Brehm K, Wolf M, Beland H, Kroner A & Frosch M (2003) Analysis of differential gene expression in *Echinococcus multilocularis* larval stages by means of spliced leader differential display. *Int J Parasitol* **33**: 1145–1159.

Budke CM, Jiamin Q, Craig PS & Torgerson PR (2005) Modeling the transmission of *Echinococcus granulosus* and *Echinococcus multilocularis* in dogs for a high endemic region of the Tibetan plateau. *Int J Parasitol* **35**: 163–170.

Buishi IE, Njoroge EM, Bouamra O & Craig PS (2005) Canine echinococcosis in northwest Libya: assessment of coproantigen ELISA, and a survey of infection with analysis of risk-factors. *Vet Parasitol* **130**: 223–232.

Bulut V, Ilhan F, Yucel AY, Onal S, Ilhan Y & Godekmerdan A (2001) Immunological follow-up of hydatid cyst cases. *Mem Inst Oswaldo Cruz* **96**: 669–671.

Cabrera M, Canova S, Rosenzvit M & Guarnera E (2002) Identification of *Echinococcus granulosus* eggs. *Diagn Microbiol Infect Dis* **44**: 29–34.

Cabrera PA, Irabedra P, Orlando D, *et al.* (2003) National prevalence of larval echinococcosis in sheep in slaughtering plants *Ovis aries* as an indicator in control programmes in Uruguay. *Acta Trop* **85**: 281–285.

Carmena D, Martinez J, Benito A & Guisantes JA (2004) Characterization of excretory-secretory products from protoscoleces of *Echinococcus granulosus* and evaluation of their potential for immunodiagnosis of human cystic echinococcosis. *Parasitology* **129**: 371–378.

Carmena D, Benito A, Martinez J & Guisantes JA (2005) Preliminary study of the presence of antibodies against excretory-secretory antigens from protoscoleces of *Echinococcus granulosus* in dogs with intestinal echinococcosis. *Mem Inst Oswaldo Cruz* **100**: 311–317.

Casulli A, La Rosa G, Manfredi MT, Di Cerbo AR, Dinkel A, Romig T, Deplazes P, Genchi C & Pozio E (2004) Copro-

diagnosis of *Echinococcus multilocularis* by a nested PCR in red foxes (*Vulpes vulpes*) from northern Italy. *Parassitologia* **46**: 419–420.

Casulli A, Manfredi MT, La Rosa G, Di Cerbo AR, Dinkel A, Romig T, Deplazes P, Genchi C & Pozio E (2005) *Echinococcus multilocularis* in red foxes (*Vulpes vulpes*) of the Italian Alpine region: is there a focus of autochthonous transmission? *Int J Parasitol* **35**: 1079–1083.

Cavagion L, Perez A, Santillan G, *et al*. (2005) Diagnosis of cystic echinococcosis on sheep farms in the south of Argentina: areas with a control program. *Vet Parasitol* **128**: 73–81.

Chemale G, van Rossum AJ, Jefferies JR, Barrett J, Brophy PM, Ferreira HB & Zaha A (2003) Proteomic analysis of the larval stage of the parasite *Echinococcus granulosus*: causative agent of cystic hydatid disease. *Proteomics* **3**: 1633–1636.

Chemale G, Ferreira HB, Barrett J, Brophy PM & Zaha A (2005) *Echinococcus granulosus* antigen B hydrophobic ligand binding properties. *Biochim Biophys Acta* **1747**: 189–194.

Chen XH, Wen H, Zhang YX, Feng XH, Lu XM & Ma D (2003) Identification of a gene engineering antibody against cystic echinococcosis in liver. *Hepatobiliary Pancreat Dis Int* **2**: 383–386.

Chow C, Gauci CG, Cowman AF & Lightowlers MW (2004) *Echinococcus granulosus:* oncosphere-specific transcription of genes encoding a host-protective antigen. *Exp Parasitol* **106**: 183–186.

Colebrook AL, Jenkins DD & Lightowlers MW (2002) Anti-parasitic effect of cyclosporin A on *Echinococcus granulosus* and characterization of the associated cyclophilin protein. *Parasitology* **125**: 485–493.

Cortez-Herrera E, Yamamoto RR, Rodrigues JJ, Farias SE, Ferreira HB & Zaha A (2001) *Echinococcus granulosus*: cloning and functional *in vitro* characterization of an actin filament fragmenting protein. *Exp Parasitol* **97**: 215–225.

Craig P (2003) *Echinococcus multilocularis*. *Curr Opin Infect Dis* **16**: 437–444.

Dai WJ, Waldvogel A, Jungi T, Stettler M & Gottstein B (2003) Inducible nitric oxide synthase deficiency in mice increases resistance to chronic infection with *Echinococcus multilocularis*. *Immunology* **108**: 238–244.

Dai WJ, Waldvogel A, Siles-Lucas M & Gottstein B (2004) *Echinococcus multilocularis* proliferation in mice and respective parasite 14-3-3 gene expression is mainly controlled by an alphabeta CD4 T-cell-mediated immune response. *Immunology* **112**: 481–488.

Dematteis S, Rottenberg M & Baz A (2003) Cytokine response and outcome of infection depends on the infective dose of parasites in experimental infection by *Echinococcus granulosus*. *Parasite Immunol* **25**: 189–197.

Deplazes P, Dinkel A & Mathis A (2003) Molecular tools for studies on the transmission biology of *Echinococcus multilocularis*. *Parasitology* **127** (Suppl), S53–S61.

Deutz A, Fuchs K, Nowotny N, Auer H, Schuller W, Stunzner D, Aspock H, Kerbl U & Kofer J (2003) Sero-epidemiological studies of zoonotic infections in hunters – comparative analysis with veterinarians, farmers, and abattoir workers. *Wien Klin Wochenschr* **115** (Suppl 3), 61–67.

Doiz O, Benito R, Gil J, Rojas A, Rubio MC & Osuna A (2002) Pre- and postsurgical detection of IgG, IgM, and IgA specific to hydatidosis by ELISA with purified antigen enriched with the 5/B antigen complex. *J Clin Lab Anal* **16**: 295–298.

Dvoroznakova E, Hrckova G, Boroskova Z, Velebny S & Dubinsky P (2004) Effect of treatment with free and liposomized albendazole on selected immunological parameters and cyst growth in mice infected with *Echinococcus multilocularis*. *Parasitol Int* **53**: 315–325.

Eckert J & De Plazes P (2004) Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev* **17**: 107–135.

Eger A, Kirch A, Manfras B, Kern P, Schulz-Key H & Soboslay PT (2003) Pro-inflammatory (IL-1beta, IL-18) cytokines and IL-8 chemokine release by PBMC in response to *Echinococcus multilocularis* metacestode vesicles. *Parasite Immunol* **25**: 103–105.

Elayoubi FA & Craig PS (2004) *Echinococcus granulosus* coproantigens: chromatographic fractionation and characterization. *Parasitology* **128**: 455–465.

Elayoubi FA, Fraser A, Jenkins DJ & Craig PS (2003) Partial characterisation of carbohydrate-rich *Echinococcus granulosus* coproantigens. *Int J Parasitol* **33**: 1553–1559.

Emery I, Leclerc C, Sengphommachanh K, Vuitton DA & Liance M (1998) In vivo treatment with recombinant IL-12 protects C57BL/6J mice against secondary alveolar echinococcosis. *Parasite Immunol* **20**: 81–91.

Fernandez C, Gregory WF, Loke P & Maizels RM (2002) Full-length-enriched cDNA libraries from *Echinococcus granulosus* contain separate populations of oligo-capped and trans-spliced transcripts and a high level of predicted signal peptide sequences. *Mol Biochem Parasitol* **122**: 171–180.

Fraize M, Sarciron ME, Azzouz S, Issaadi N, Bosquet G & Petavy AF (2005) Immunogenicity of two *Echinococcus granulosus* antigens EgA31 and EgTrp in mice. *Parasitol Res* **96**: 113–120.

Freire T, Fernandez C, Chalar C, Maizels RM, Alzari P, Osinaga E & Robello C (2004) Characterization of a UDP-*N*-acetyl-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase with an unusual lectin domain from the platyhelminth parasite *Echinococcus granulosus*. *Biochem J* **382**: 501–510.

Fujimoto Y, Ito A, Ishikawa Y, Inoue M, Suzuki Y, Ohhira M, Ohtake T & Kohgo Y (2005) Usefulness of recombinant Em18-ELISA to evaluate efficacy of treatment in patients with alveolar echinococcosis. *J Gastroenterol* **40**: 426–431.

Gauci C, Heath D, Chow C & Lightowlers MW (2005) Hydatid disease: vaccinology and development of the EG95 recombinant vaccine. *Expert Rev Vaccines* **4**: 103–112.

Gelmedin V, Zavala-Gongora R, Fernandez C & Brehm K (2005) *Echinococcus multilocularis*: Cloning and characterization of a member of the SNW/SKIP family of transcriptional coregulators. *Exp Parasitol* **111**: 115–120.

Gimba ER, Chemale G, Farias SS & Zaha A (2000) Cloning and characterization of *Echinococcus granulosus* (Cestode) EgactI

and EgactII actin gene promoters and their functional analysis in the NIH3T3 mouse cell line. *Braz J Med Biol Res* **33**: 1283–1292.

Godot V, Harraga S, Podoprigora G, Liance M, Bardonnet K & Vuitton DA (2003) IFN alpha-2a protects mice against a helminth infection of the liver and modulates immune responses. *Gastroenterology* **124**: 1441–1450.

Gonzalez-Sapienza G & Cachau RE (2003) Identification of critical residues of an immunodominant region of *Echinococcus granulosus* antigen B. *J Biol Chem* **278**: 20179–20184.

Gottstein B (1992) Molecular and immunological diagnosis of echinococcosis. *Clin Microbiol Rev* **5**: 248–261.

Gottstein B, Jacquier P, Bresson-Hadni S & Eckert J (1993) Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus antigen. *J Clin Microbiol* **31**: 373–376.

Grenard P, Bresson-Hadni S, El Alaoui S, Chevallier M, Vuitton DA & Ricard-Blum S (2001) Transglutaminase-mediated cross-linking is involved in the stabilization of extracellular matrix in human liver fibrosis. *J Hepatol* **35**: 367–375.

Haag KL, Zanotto P, Alves-Junior L, Gasser RB, Zaha A & Ayala FJ (2006) Searching for antigen B genes and their adaptive sites in distinct starains and species of the helminth *Echinococcus*. *Infect Genet Evol*, in press.

Hernandez A, Cardozo G, Dematteis S, *et al.* (2005) Cystic echinococcosis: analysis of the serological profile related to the risk factors in individuals without ultrasound liver changes living in an endemic area of Tacuarembo, Uruguay. *Parasitology* **130**: 455–460.

Hofer S, Gloor S, Muller U, Mathis A, Hegglin D & Deplazes P (2000) High prevalence of *Echinococcus multilocularis* in urban red foxes (*Vulpes vulpes*) and voles (Arvicola terrestris) in the city of Zurich, Switzerland. *Parasitology* **120**: 135–142.

Hubert K, Cordero E, Frosch M & Solomon F (1999) Activities of the EM10 protein from *Echinococcus multilocularis* in cultured mammalian cells demonstrate functional relationships to ERM family members. *Cell Motil Cytoskeleton* **42**: 178–188.

Hubert K, Zavala-Gongora R, Frosch M & Brehm K (2004) Identification and characterization of PDZ-1, a N-ERMAD specific interaction partner of the *Echinococcus multilocularis* ERM protein Elp. *Mol Biochem Parasitol* **134**: 149–154.

Ibrahem MM, Rafiei A, Dar FK, Azwai SM, Carter SD & Craig PS (2002) Serodiagnosis of cystic echinococcosis in naturally infected camels. *Parasitology* **125**: 245–251.

Ito A, Schantz PM & Wilson JF (1995) Em18, a new serodiagnostic marker for differentiation of active and inactive cases of alveolar hydatid disease. *Am J Trop Med Hyg* **52**: 41–44.

Ito A, Xiao N, Liance M, *et al.* (2002) Evaluation of an enzyme-linked immunosorbent assay (ELISA) with affinity-purified Em18 and an ELISA with recombinant Em18 for differential diagnosis of alveolar echinococcosis: results of a blind test. *J Clin Microbiol* **40**: 4161–4165.

Jiang L, Xu XN, Li X, Xue HC & Feng Z (2004) Identification of the immunodominant regions of the Em18 antigen and improved serodiagnostic specificity for alveolar echinococcosis. *Parasite Immunol* **26**: 377–385.

Kato N, Nonaka N, Oku Y & Kamiya M (2005a) Modified cellular immune responses in dogs infected with *Echinococcus multilocularis*. *Parasitol Res* **95**: 339–345.

Kato N, Nonaka N, Oku Y & Kamiya M (2005b) Immune responses to oral infection with *Echinococcus multilocularis* protoscoleces in gerbils: modified lymphocyte responses due to the parasite antigen. *Parasitol Res* **96**: 12–17.

Kittelberger R, Reichel MP, Jenner J, Heath DD, Lightowlers MW, Moro P, Ibrahem MM, Craig PS & O'Keefe JS (2002) Evaluation of three enzyme-linked immunosorbent assays (ELISAs) for the detection of serum antibodies in sheep infected with *Echinococcus granulosus*. *Vet Parasitol* **110**: 57–76.

Konrad C, Kroner A, Spiliotis M, Zavala-Gongora R & Brehm K (2003) Identification and molecular characterisation of a gene encoding a member of the insulin receptor family in *Echinococcus multilocularis*. *Int J Parasitol* **33**: 301–12.

Lahmar S, Kilani M & Torgerson PR (2001) Frequency distributions of *Echinococcus granulosus* and other helminths in stray dogs in Tunisia. *Ann Trop Med Parasitol* **95**: 69–76.

Lawn SD, Bligh J, Craig PS & Chiodini PL (2004) Human cystic echinococcosis: evaluation of post-treatment serologic follow-up by IgG subclass antibody detection. *Am J Trop Med Hyg* **70**: 329–335.

Lightowlers MW & Heath DD (2004) Immunity and vaccine control of *Echinococcus granulosus* infection in animal intermediate hosts. *Parassitologia* **46**: 27–31.

Li FR, Shi YE, Shi DZ, Vuitton DA & Craig PS (2003) Kinetic analysis of cytokines and immunoglobulin G subclass in BALB/c mice infected with *Echinococcus* alveolaris. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **21**: 357–360.

Li J, Zhang WB, Loukas A, Lin RY, Ito A, Zhang LH, Jones M & McManus DP (2004) Functional expression and characterization of *Echinococcus granulosus* thioredoxin peroxidase suggests a role in protection against oxidative damage. *Gene* **326**: 157–165.

Li J, Zhang WB & McManus DP (2004) Recombinant antigens for immunodiagnosis of cystic echinococcosis. *Biol Proced Online* **6**: 67–77.

Li J, Zhang WB, Wilson M, Ito A & McManus DP (2003) A novel recombinant antigen for immunodiagnosis of human cystic echinococcosis. *J Infect Dis* **188**: 1951–1960.

Lopera L, Moro PL, Chavez A, Montes G, Gonzales A & Gilman RH (2003) Field evaluation of a coproantigen enzyme-linked immunosorbent assay for diagnosis of canine echinococcosis in a rural Andean village in Peru. *Vet Parasitol* **117**: 37–42.

Lorenzo C, Salinas G, Brugnini A, Wernstedt C, Hellman U & Gonzalez-Sapienza G (2003a) *Echinococcus granulosus* antigen 5 is closely related to proteases of the trypsin family. *Biochem J* **369**: 191–198.

Lorenzo C, Salinas G, Brugnini A, Wernstedt C, Hellman U & Gonzalez-Sapienza G (2003b) *Echinococcus granulosus* antigen

5 is closely related to proteases of the trypsin family. *Biochem J* **369**: 191–198.

Lorenzo C, Ferreira HB, Monteiro KM, *et al*. (2005) Comparative analysis of the diagnostic performance of six major *Echinococcus granulosus* antigens assessed in a double-blind, randomized multicenter study. *J Clin Microbiol* **43**: 2764–2770.

Lorenzo C, Last JA & Gonzalez-Sapienza GG (2005) The immunogenicity of *Echinococcus granulosus* antigen 5 is determined by its post-translational modifications. *Parasitology* **131**: 669–677.

Losson B, Kervyn T, Detry J, Pastoret PP, Mignon B & Brochier B (2003) Prevalence of *Echinococcus multilocularis* in the red fox (*Vulpes vulpes*) in southern Belgium. *Vet Parasitol* **117**: 23–28.

Macpherson CN, Kachani M, Lyagoubi M, Berrada M, Shepherd M, Fields PF & El Hasnaoui M (2004) Cystic echinococcosis in the Berber of the Mid Atlas mountains, Morocco: new insights into the natural history of the disease in humans. *Ann Trop Med Parasitol* **98**: 481–490.

Mahmoud MS & Abou Gamra MM (2004) Alkaline phosphatase from *Echinococcus granulosus* metacestodes for immunodiagnosis of human cystic echinococcosis. *J Egypt Soc Parasitol* **34**: 865–879.

Mamuti W, Yamasaki H, Sako Y, *et al*. (2004) Molecular cloning, expression, and serological evaluation of an 8-kilodalton subunit of antigen B from *Echinococcus multilocularis*. *J Clin Microbiol* **42**: 1082–1088.

Manderson D, Dempster R & Chisti Y (2005) A recombinant vaccine against hydatidosis: production of the antigen in *Escherichia coli*. *J Ind Microbiol Biotechnol*, 1–10.

Manfras BJ, Reuter S, Wendland T & Kern P (2002) Increased activation and oligoclonality of peripheral CD8(+) T cells in the chronic human helminth infection alveolar echinococcosis. *Infect Immun* **70**: 1168–1174.

Marsland BJ, Tisdall DJ, Heath DD & Mercer AA (2003) Construction of a recombinant orf virus that expresses an *Echinococcus granulosus* vaccine antigen from a novel genomic insertion site. *Arch Virol* **148**: 555–562.

McManus DP (2006) Molecular discrimination of taeniid cestodes. *Parasitol Int*, in press.

McManus DP, Zhang W, Li J & Bartley PB (2003) Echinococcosis. *Lancet* **362**: 1295–1304.

Merckelbach A, Wager M & Lucius R (2003) Analysis of cDNAs coding for immunologically dominant antigens from an oncosphere-specific cDNA library of *Echinococcus multilocularis*. *Parasitol Res* **90**: 493–501.

Moreno M, Benavidez U, Carol H, Rosenkranz C, Welle M, Carmona C, Nieto A & Chabalgoity JA (2004) Local and systemic immune responses to *Echinococcus granulosus* in experimentally infected dogs. *Vet Parasitol* **119**: 37–50.

Moro PL, Garcia HH, Gonzales AE, Bonilla JJ, Verastegui M & Gilmanmd RH (2005) Screening for cystic echinococcosis in an endemic region of Peru using portable ultrasonography and the enzyme-linked immunoelectrotransfer blot (EITB) assay. *Parasitol Res* **96**: 242–246.

Moro PL, Lopera L, Bonifacio N, Gonzales A, Gilman RH & Moro MH (2005) Risk factors for canine echinococcosis in an endemic area of Peru. *Vet Parasitol* **130**: 99–104.

Myjak P, Nahorski W, Pietkiewicz H, von Nickisch-Rosenegk M, Stolarczyk J, Kacprzak E, Felczak-Korzybska I, Szostakowska B & Lucius R (2003) Molecular confirmation of human alveolar echinococcosis in Poland. *Clin Infect Dis* **37**: e121–e125.

Naidich A, McManus DP, Canova SG, Gutierrez AM, Zhang WB, Eduardo A, Guarnera EA & Rosenzvit MC (2006) Patent and pre-patent detection of *Echinococcus granulosus* genotypes in the definitive host. *Mol Cell Probes*, in press.

Nasrieh MA & Abdel-Hafez SK (2004) *Echinococcus granulosus* in Jordan: assessment of various antigenic preparations for use in the serodiagnosis of surgically confirmed cases using enzyme immuno assays and the indirect haemagglutination test. *Diagn Microbiol Infect Dis* **48**: 117–123.

Ortona E, Margutti P, Delunardo F, Vaccari S, Rigano R, Profumo E, Buttari B, Teggi A & Siracusano A (2003) Molecular and immunological characterization of the C-terminal region of a new *Echinococcus granulosus* Heat Shock Protein 70. *Parasite Immunol* **25**: 119–126.

Pearce EJ & MacDonald AS (2002) The immunobiology of schistosomiasis. *Nat Rev Immunol* **2**: 499–511.

Qaqish AM, Nasrieh MA, Al-Qaoud KM, Craig PS & Abdel-Hafez SK (2003) The seroprevalences of cystic echinococcosis, and the associated risk factors, in rural-agricultural, bedouin and semi-bedouin communities in Jordan. *Ann Trop Med Parasitol* **97**: 511–520.

Rehmann P, Grone A, Lawrenz A, Pagan O, Gottstein B & Bacciarini LN (2003) *Echinococcus multilocularis* in two lowland gorillas (Gorilla g. gorilla). *J Comp Pathol* **129**: 85–88.

Rehmann P, Grone A, Gottstein B, Vollm J, Sager H, Janovsky M & Bacciarini LN (2005) Detection of *Echinococcus multilocularis* infection in a colony of cynomolgus monkeys (Macaca fascicularis) using serology and ultrasonography. *J Vet Diagn Invest* **17**: 183–186.

Reiterova K, Miterpakova M, Turcekova L, Antolova D & Dubinsky P (2005) Field evaluation of an intravital diagnostic test of *Echinococcus multilocularis* infection in red foxes. *Vet Parasitol* **128**: 65–71.

Ricard-Blum S, Bresson-Hadni S, Guerret S, Grenard P, Volle PJ, Risteli L, Grimaud JA & Vuitton DA (1996) Mechanism of collagen network stabilization in human irreversible granulomatous liver fibrosis. *Gastroenterology* **111**: 172–182.

Rigano R, Profumo E, Ioppolo S, Notargiacomo S, Ortona E, Teggi A & Siracusano A (1995) Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. *Clin Exp Immunol* **102**: 281–285.

Rigano R, Profumo E, Ioppolo S, Notargiacomo S, Teggi A & Siracusano A (1999) Serum cytokine detection in the clinical follow up of patients with cystic echinococcosis. *Clin Exp Immunol* **115**: 503–507.

Rigano R, Buttari B, De Falco E, Profumo E, Ortona E, Margutti P, Scotta C, Teggi A & Siracusano A (2004) *Echinococcus granulosus*-specific T-cell lines derived from patients at various

clinical stages of cystic echinococcosis. *Parasite Immunol* **26**: 45–52.

Rogan MT, Craig PS, Zehyle E, Masinde G, Wen H & Zhou P (1992) In vitro killing of taeniid oncospheres, mediated by human sera from hydatid endemic areas. *Acta Trop* **51**: 291–296.

Rosenzvit MC, Zhang WB, Motazedian H, Smyth D, Pearson M, Loukas A, Jones MK & McManus DP (2006) Identification of membrane-bound and secreted proteins from *Echinococcus granulosus* by signal sequence trap. *Int J Parasitol* **36**: 123–130.

Sako Y, Nakao M, Nakaya K, Yamasaki H, Gottstein B, Lightowers MW, Schantz PM & Ito A (2002) Alveolar echinococcosis: characterization of diagnostic antigen Em18 and serological evaluation of recombinant Em18. *J Clin Microbiol* **40**: 2760–2765.

Sanchez Thevenet P, Jensen O, Mellado I, Torrecillas C, Raso S, Flores ME, Minvielle MC & Basualdo JA (2003) Presence and persistence of intestinal parasites in canine fecal material collected from the environment in the Province of Chubut, Argentine Patagonia. *Vet Parasitol* **117**: 263–269.

Shaikenov BS, Rysmukhambetova AT, Massenov B, Deplazes P, Mathis A & Torgerson PR (2004) Short report: the use of a polymerase chain reaction to detect *Echinococcus granulosus* (G1 strain) eggs in soil samples. *Am J Trop Med Hyg* **71**: 441–443.

Siles-Lucas M, Merli M, Mackenstedt U & Gottstein B (2003) The *Echinococcus multilocularis* 14-3-3 protein protects mice against primary but not secondary alveolar echinococcosis. *Vaccine* **21**: 431–439.

Simsek S & Koroglu E (2004) Evaluation of enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) for immunodiagnosis of hydatid diseases in sheep. *Acta Trop* **92**: 17–24.

Siracusano A, Buttari B, Delunardo F, Profumo E, Margutti P, Ortona E, Rigano R & Teggi A (2004) Critical points in the immunodiagnosis of cystic echinococcosis in humans. *Parassitologia* **46**: 401–403.

Spiliotis M & Brehm K (2004) *Echinococcus multilocularis*: identification and molecular characterization of a Ral-like small GTP-binding protein. *Exp Parasitol* **107**: 163–172.

Spiliotis M, Kroner A & Brehm K (2003) Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm *Echinococcus multilocularis*. *Gene* **323**: 57–65.

Spiliotis M, Tappe D, Bruckner S, Mosch HU & Brehm K (2005) Molecular cloning and characterization of Ras- and Raf-homologues from the fox-tapeworm *Echinococcus multilocularis*. *Mol Biochem Parasitol* **139**: 225–237.

Stefanic S, Shaikenov BS, Deplazes P, Dinkel A, Torgerson PR & Mathis A (2004) Polymerase chain reaction for detection of patent infections of *Echinococcus granulosus* ("sheep strain") in naturally infected dogs. *Parasitol Res* **92**: 347–351.

Torgerson PR (2006) Canid immunity to *Echinococcus* spp: impact on transmission. *Parasite Immunol*, in press.

Torgerson PR, Shaikenov BS, Rysmukhambetova AT, Ussenbayev AE, Abdybekova AM & Burtisurnov KK (2003) Modelling the transmission dynamics of *Echinococcus granulosus* in dogs in rural Kazakhstan. *Parasitology* **126**: 417–424.

Varcasia A, Garippa G & Scala A (2004) The diagnosis of *Echinococcus granulosus* in dogs. *Parassitologia* **46**: 409–412.

Virginio VG, Hernandez A, Rott MB, Monteiro KM, Zandonai AF, Nieto A, Zaha A & Ferreira HB (2003) A set of recombinant antigens from *Echinococcus granulosus* with potential for use in the immunodiagnosis of human cystic hydatid disease. *Clin Exp Immunol* **132**: 309–315.

Vuitton DA (2004) Echinococcosis and allergy. *Clin Rev Allergy Immunol* **26**: 93–104.

Vuitton DA, Bresson-Hadni S, Laroche L, Kaiserlian D, Guerret-Stocker S, Bresson JL & Gillet M (1989) Cellular immune response in *Echinococcus multilocularis* infection in humans. II. Natural killer cell activity and cell subpopulations in the blood and in the periparasitic granuloma of patients with alveolar echinococcosis. *Clin Exp Immunol* **78**: 67–74.

Wang Y, Zhang X, Bartholomot B, *et al*. (2003) Classification, follow-up and recurrence of hepatic cystic echinococcosis using ultrasound images. *Trans R Soc Trop Med Hyg* **97**: 203–211.

Wei XL, Ding JB, Xu Y, Wen H & Lin RY (2004) Change of cytokines in mice with *Echinococcus multilocularis* infection. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **22**: 361–364.

Wynn TA, Thompson RW, Cheever AW & Mentink-Kane MM (2004) Immunopathogenesis of schistosomiasis. *Immunol Rev* **201**: 156–167.

Xu MQ, Zhu B, Xue HC, Li X, Guo XR, Zhang YH & Li H (2002) Reexamination of specific antibodies in sera of cystic echinococccosis patients with IgG negative seroresponse. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **20**: 148–151.

Yimam AE, Nonaka N, Oku Y & Kamiya M (2002) Prevalence and intensity of *Echinococcus multilocularis* in red foxes (*Vulpes vulpes* schrencki) and raccoon dogs (Nyctereutes procyonoides albus) in Otaru City, Hokkaido, Japan. *Jpn J Vet Res* **49**: 287–296.

Zavala-Gongora R, Kroner A, Wittek B, Knaus P & Brehm K (2003) Identification and characterisation of two distinct Smad proteins from the fox-tapeworm *Echinococcus multilocularis*. *Int J Parasitol* **33**: 1665–1677.

Zhang W, You H, Zhang Z, Turson G, Hasyet A & McManus DP (2001) Further studies on an intermediate host murine model showing that a primary *Echinococcus granulosus* infection is protective against subsequent oncospheral challenge. *Parasitol Int* **50**: 279–283.

Zhang W, Li J & McManus DP (2003a) Concepts in immunology and diagnosis of hydatid disease. *Clin Microbiol Rev* **16**: 18–36.

Zhang W, Li J, You H, Zhang Z, Turson G, Loukas A & McManus DP (2003b) Short report: *Echinococcus granulosus* from Xinjiang, PR China: cDNAS encoding the EG95 vaccine

antigen are expressed in different life cycle stages and are conserved in the oncosphere. *Am J Trop Med Hyg* **68**: 40–43.

Zhang W, Li J, You H, Zhang Z, Turson G, Loukas A & McManus DP (2003c) A gene family from *Echinococcus granulosus* differentially expressed in mature adult worms. *Mol Biochem Parasitol* **126**: 25–33.

Zhang W, You H, Li J, Zhang Z, Turson G, Aili H, Wang J & McManus DP (2003d) Immunoglobulin profiles in a murine intermediate host model of resistance for *Echinococcus granulosus* infection. *Parasite Immunol* **25**: 161–168.

Zhang WB (2003) *Echinococcus granulosus*: studies of protective immunity, and the isolation and characterisation of stage-specific genes. PhD thesis,University of Queensland, Brisbane.

Zhang WB & Zhao DZ (1992) A comparison of calcification of hydatid cysts in sheep pasturred and post-feeded. *Chinese J Vet Sci Technol* **22**: 28–29 (in Chinese).